

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
8 March 2001 (08.03.2001)

PCT

(10) International Publication Number  
WO 01/16349 A1(51) International Patent Classification: C12P 19/18.  
C12N 9/26 // A23L 1/09, D21H 19/54

(21) International Application Number: PCT/DK00/00461

(22) International Filing Date: 21 August 2000 (21.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
PA 1999 01220 1 September 1999 (01.09.1999) DK  
PA 2000 00035 12 January 2000 (12.01.2000) DK(71) Applicant: NOVOZYMES A/S (DK/DK); Patents,  
Krogshoejvej 36, DK-2880 Bagsvaerd (DK).(72) Inventors: PEDERSEN, Sven; Emil Reesens Vej  
9, DK-2820 Gentofte (DK); VANG HENDRIKSEN,  
Hanne; Vejlemosevej 5A, DK-2840 Høje (DK).(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

## Published:

--- With international search report.

--- Before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments.For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR PRODUCTION OF MALTOSE AND/OR ENZYMATICALLY MODIFIED STARCH

(57) Abstract: The present invention relates to a method for preparing maltose and/or modified starch, wherein starch is treated with a variant of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1. The treatment can be carried out at elevated temperatures compared to prior art processes due to the increased thermostability of the maltogenic amylase variants. The method is simple and cheap and gives rise to a maltose of high purity and to a cheap modified starch product useable, e.g., as a fat replacer in foods.

WO 01/16349 A1

METHOD FOR PRODUCTION OF MALTOSE AND/OR ENZYMATICALLY MODIFIED STARCH

TECHNICAL FIELD

5 This invention is concerned with a method for production of maltose and/or an enzymatically modified starch, whereby starch (either raw starch or gelatinized starch) is treated with a maltogenic amylase variant. In this context maltose syrup covers various types of maltose syrups, such as Low Maltose Syrup, High  
10 Maltose Syrup, and Ultra High Maltose Syrup, as will be described further below. The invention also relates to producing speciality syrups, and sizing of paper.

BACKGROUND OF THE INVENTION

15 Maltose is a disaccharide, which is used in huge amounts in the candy industry. Maltose does not crystallize easily, in contradistinction to, e.g., glucose, which is able to crystallize even in the presence of impurities in high concentrations. Maltose is not able to crystallize and thus to be purified  
20 further, unless the maltose used as a starting material exhibits a purity above 90%. Also, the fact that maltose does not crystallize easily is one of the reasons why maltose is a valuable raw material, e.g., in the candy industry. Usually, starches such as corn, potato, wheat, manioc and rice starch are  
25 used as the starting material in commercial large scale production of sugars, such as high fructose syrup, high maltose syrup, maltodextrins, amylose, G4-G6 oligosaccharides and other carbohydrate products such as fat replacers.

30 Maltose has also other applications, e.g., as the active component of intravenous injection liquids intended for provision of sugar for the patient and as a component in frozen deserts (due to the fact that the crystallization ability of maltose is very little), in the baking and brewing industry, and for  
35 production of maltitol, which can be used as a sweetening agent, like sorbitol, vide Glycose Sirups, Science and Technology, Elsevier Applied Science Publishers 1984, pages 117 - 135.

Below is given a summary of the process steps commonly used in the production of maltose:

#### Degradation of starch

5 Starch usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains  $\alpha$ -1,4 D-glucose residues are joined by  $\alpha$ -1,6 glucosidic linkages. Amylopectin is partially degraded by  $\alpha$ -amylase, which hydrolyzes the 1,4- $\alpha$ -glucosidic linkages to  
10 produce branched and linear oligosaccharides. Prolonged degradation of amylopectin by  $\alpha$ -amylase results in the formation of so-called  $\alpha$ -limit dextrins which are not susceptible to further hydrolysis by the  $\alpha$ -amylase. Branched oligosaccharides can be hydrolyzed into linear oligosaccharides by a debranching  
15 enzyme. The remaining branched oligosaccharides can be depolymerized to D-glucose by glucoamylase, which hydrolyzes linear oligosaccharides into D-glucose.

Amylose is a linear polyaccharide built up of D-glucopyranose  
20 units linked together by  $\alpha$ -1,4 glucosidic linkages. Amylose is degraded into shorter linear oligosaccharides by  $\alpha$ -amylase, the linear oligosaccharides being depolymerized into D-glucose by glucoamylase.

25 In the case of converting starch into a sugar, the starch is depolymerized. The depolymerization process consists of a pretreatment step and two or three consecutive process steps, namely a liquefaction process, a saccharification process and, depending on the desired end product, optionally an  
30 isomerization process.

#### Pre-treatment of native starch

Native starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch  
35 slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in

viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation.

5

#### Liquefaction

During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an  $\alpha$ -amylase (e.g. Termamyl™, available from Novo Nordisk A/S, Denmark). The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The pH generally lies between about 5.5 and 6.2. In order to ensure an optimal enzyme stability under these conditions, calcium is added, e.g. 1 mM of calcium (40 ppm free calcium ions). After this treatment the liquefied starch will have a "dextrose equivalent" (DE) of 10-15.

#### Saccharification

After the liquefaction process the maltodextrins are converted into maltose by addition of a  $\beta$ -amylase and a debranching enzyme, such as an isoamylase (see e.g. US Patent No. 4,335,208) or a pullulanase (e.g. Promozyme™, available from Novo Nordisk A/S) (see US Patent No. 4,560,651). Before this step the pH is reduced to a value below 4.5, e.g. about 3.8, maintaining the high temperature (above 95°C) for a period of e.g. about 30 min. to inactivate the liquefying  $\alpha$ -amylase to reduce the formation of short oligosaccharides called "panose precursors" which cannot be hydrolyzed properly by the debranching enzyme.

The temperature is then lowered to 60°C,  $\beta$ -amylase and debranching enzyme are added, and the saccharification process proceeds for about 24-72 hours.

Normally, when denaturing the  $\alpha$ -amylase after the liquefaction step, a small amount of the product comprises panose precursors which cannot be degraded by pullulanases. If active amylase from

the liquefaction step is present during saccharification (i.e. no denaturing), this level can be as high as 1-2% or even higher, which is highly undesirable as it lowers the saccharification yield significantly. For this reason, it is also preferred that the  $\alpha$ -amylase is one which is capable of degrading the starch molecules into long, branched oligosaccharides (such as, e.g., the Fungamyl™-like  $\alpha$ -amylases) rather than shorter branched oligosaccharides.

It will be apparent from the above discussion that the known starch conversion processes are performed in a series of steps, due to the different requirements of the various enzymes in terms of e.g. temperature and pH. It would therefore be desirable to be able to engineer one or more of these enzymes so that the overall process could be performed in a more economical and efficient manner. One possibility in this regard is to substitute (or reduce the amount of) the  $\beta$ -amylase used in the saccharification step with a maltogenic amylase (vide infra) with a higher thermostability than the commonly used  $\beta$ -amylases.

The present invention relates to a process for converting starch using such thermostable maltogenic amylases, which provides a number of important advantages which will be discussed in detail below.

A method for producing maltose and a limit dextrin is disclosed in WO 95/10627.

#### SUMMARY OF THE INVENTION

In a first aspect the present invention relates to a method for preparing maltose and/or a modified starch comprising the following steps:

I) treating starch with a variant of a maltogenic amylase having the amino acid sequence shown in position 1-586 of SEQ ID NO: 1, where the variant

- a) has maltogenic amylase activity;
- b) has at least 70% identity to position 1-686 of SEQ ID NO: 1, and
- c) has optimum maltogenic amylase activity in the pH range 3.5-7.0; and

II) optionally recovering the maltose and/or the modified starch.

10 In a second aspect the present invention relates to a modified starch obtainable by the method according to the invention.

In a third aspect the present invention relates to a process for surface-sizing and/or coating paper, wherein paper is treated  
15 with an aqueous size or coating liquid that contains the modified starch of the invention.

In a fourth aspect the present invention relates to paper obtainable by the process of the invention.

20

In a fifth aspect the present invention relates to a food product comprising an emulsion containing the modified starch of the invention.

25 In a sixth aspect the present invention relates to a beverage flavour concentrate comprising an emulsion containing the modified starch of the invention.

In a seventh aspect the present invention relates to a flavouring agent comprising an emulsion containing the modified  
30 starch of the invention.

#### DETAILED DESCRIPTION OF THE INVENTION

When used in the present context, the term "modified starch" is  
35 intended to cover starch, which has been subjected to a maltogenic amylase treatment.

Enzymatic treatment of starch without gelatinization

In one aspect the purpose of the invention is the provision of a method for production of maltose, for producing a high maltose syrup with a purity of more than 90%, preferably 95%, especially 5 97%, even more preferred 99%, which is simpler and cheaper in comparison to the prior art methods for production of maltose.

In one interesting embodiment of the invention, the starch is treated with the maltogenic amylase variant at a temperature 10 which is below the lowest temperature at which raw starch is gelatinized, i.e. the starch to be treated is raw starch, such as raw waxy maize starch. Typical treatment temperatures are above 50°C, such as in the range of from 50 to 80°C, dependent on the temperature optimum of the enzyme. Preferably the temperature is 15 above 60°C, e.g. in the range of from 60 to 80°C, more preferably the temperature is above 65°C, such as in the range of from 65 to 75°C, e.g. in the range of from 65 to 70°C.

Dependent on the actual purpose of the process, i.e. whether the 20 practitioner desires to produce maltose or a modified starch, which in turn may be used as a fat replacer or as a sizing agent, the reaction conditions, typically the reaction time, are adjusted accordingly. If, for example, maltose is the desired product (which is most often the case), the reaction is continued 25 until the limit dextrin is obtained (see above), i.e. in one interesting embodiment of the invention the modified starch is a limit dextrin.

Independent of whether the reaction actually produces a modified 30 starch which is, or which is not, a limit dextrin, the produced maltose and/or the modified starch may be recovered from the reaction mixture.

In one preferred embodiment of the invention the recovery is 35 performed by ultrafiltration. However, other methods commonly used for separating disaccharides from larger oligo- or polysaccharides may be used and will be well known to the person

skilled in the art.

In the ultrafiltration step, the maltose is in the permeate, and the modified starch (e.g. in the form of a limit dextrin) is produced as the solid phase by liquid-solid separation of the retentate.

As indicated, the recovery of maltose is carried out as follows. After treatment of the raw starch the solid phase and the supernatant therefrom comprising mainly oligosaccharides and maltose is subjected to an ultrafiltration, which yields a permeate, the dry matter of which contains more than 90%, preferably more than 95%, more preferably 97%, even more preferably more than 99% of maltose. Centrifugation or filtration of the unreacted raw starch can be carried out in a step before the ultrafiltration step, if wanted. In a preferred embodiment, the ultrafiltration step is carried out simultaneously with the treatment of the raw starch with the maltogenic amylase variant, and the temperature is above 40°C. In this manner the process time can be reduced, and also, the yield of the maltose in the permeate is improved.

It is the recognition that maltogenic amylase variants, which is able to give rise to a degradation product of raw starch, which consists of a mixture of maltose and high molecular oligosaccharides, which mixture by simple ultrafiltration gives rise to a permeate with a dry matter consisting of more than 90%, preferably more than 95%, more preferably 97%, even more preferably more than 99% of maltose.

30

It goes without saying that the pH during the method should be at or in the vicinity of the pH optimum of the maltogenic amylase variant used for the production of maltose.

35 Due to, e.g., the higher thermostability of the contemplated maltogenic amylase variants the method of the invention can be carried out at higher temperatures. This is evidently advantageous.

Also, according to the invention it has been found that the modified starch, which *prima facie* would be considered a waste product has applicability as a fat replacer in foods. Moreover, 5 it is contemplated that the modified starch has applicability as a sizing agent for surface-sizing and/or coating of paper. Other applications include emulsions containing the modified starch, food products containing such emulsions as well as beverage flavour concentrates and flavouring agents containing such 10 emulsions.

A preferred embodiment of the method according to the invention is characterized by the fact that the raw starch is waxy maize starch. With waxy maize starch a high yield is obtained, and 15 also, the reaction proceeds smoothly, due to the fact that the viscosity of the reaction mixture is low.

Specifically contemplated are maltogenic amylase variants of the maltogenic amylase having the amino acid sequence shown as amino 20 acids 1-686 of SEQ ID NO: 1 and this particular enzyme has been described in the paper "Properties and application of a thermostable, maltogenic amylase, produced by a strain of *Bacillus* modified by recombinant-DNA techniques" by Helle Outtrup and Barrie E. Norman of Novo Nordisk A/S, *Die Stärke*, 36 (1984), 25 405-411. No maltogenic amylase variants are mentioned. Variants contemplated for used in any of the methods of the invention are described more detailed below.

#### Enzymatic treatment of starch with galarinization

30 In a second interesting embodiment of the invention the starch is gelatinized before the enzymatic treatment step. Due to the contemplated higher thermostability of the maltogenic amylase variants, it is envisaged that the treatment with the maltogenic amylase variant may be performed at a temperature above 55°C, in 35 particular above 70°C, such as above 75°C, e.g. above 80°C, preferably above 85°C, more preferably above 90°C, such as above 95°C.

Thus, as will be acknowledged by the skilled person, this opens up the possibility that the treatment with the maltogenic amylase variant can be carried out during the liquefaction step.

5 Obviously, the treatment with the maltogenic amylase variant may also be carried out after the liquefaction step, e.g. during the saccharification step.

In a similar way as explained above, the desired products, i.e. the maltose and/or the modified starch (or optionally the limit  
10 dextrin) may be recovered by methods known in the art. In a preferred embodiment, the recovery is carried out by ultrafiltration as explained above.

15 Also, the invention comprises the modified starch, e.g. the limit dextrin, prepared as in the method according to the invention. If for some reason, the limit dextrin in a specific context is the important product, and the maltose is of no significance, the ultrafiltration is unnecessary, as the limit dextrin can be  
20 produced directly after the amylolytic degradation by solid-liquid separation of the amylolytic degradation mixture and by washing of the solid phase. Besides being characterized by the fact that it is produced by means of the method according to the invention the limit dextrin according to the invention is  
25 characterized by the fact that the ratio  $\alpha$ -1,4 bonds/ $\alpha$ -1,6 bonds is smaller than for the raw starch (which is consistent with the assumption that the special amylase only cleaves the  $\alpha$ -1,4 bonds of the starch). Due to the fact that the limit dextrin according to the invention *prima facie* would be considered a waste product,  
30 it is very cheap.

The invention will be explained in further details below, where a detailed description of the process conditions, etc. when preparing various types of maltose syrups, are explained. In  
35 general, the content of dry substance (DS) in the starch slurry is between 10-30%, preferably 20-25%. The DS content in relation to this invention advantageously can have a value between 10-50%, preferably between 20-40%.

### Maltose Syrup production using maltogenic amylase variants

According to the invention maltogenic amylase variants (described further below) may advantageously be used for producing a number of maltose syrup products, such as a Low Maltose Syrup, a High Maltose Syrup, and an Ultra High Maltose Syrup.

#### Production of Low Maltose Syrup (containing 33-37% maltose)

To produce Low Maltose Syrup starch is liquefied to a DE of 10-20. The temperature and pH of the liquefied starch is adjusted to about 70°C and a pH of about 5.0, respectively, and is subjected to maltogenic amylase variant activity (e.g., 4000 MANU/g, 150 ml/t DS) and  $\alpha$ -amylase activity (e.g., Termamyl™ 120 L, 200 g/t DS) for 18-42 hours. The process time depends on the desired saccharide spectrum to be achieved.

The dose of  $\alpha$ -amylase activity (e.g., Termamyl™) influences the level of dextrose and maltotriose, i.e. a higher dosage results in higher level. Further, the dose of the maltogenic amylase activity influences the composition so that a higher dosage results in higher dextrose and maltose levels, but a lower maltotriose level.

#### Production of High Maltose Syrup (containing 50-55% maltose)

To produce High Maltose Syrup starch is liquefied to DE 10-20. The pH and temperature of the liquefied starch is adjusted to 65°C and to a pH around 5.0, respectively, and is subjected to maltogenic amylase activity (e.g., 4000 MANU/g, 0.4 l/t DS), pullulanase activity (e.g., Promozyme™ 600 L, 0.3 l/t DS) and  $\alpha$ -amylase activity (e.g., BAN 240 L or Termamyl™ 120 L, type LS, 0.4 kg/t DS) for 24-41 hours. The specific process time depends on the desired saccharide spectrum to be achieved. By increasing the dosage of the maltogenic amylase and pullulanase the maltose content can be increased.

Alternatively a High Maltose Syrup may be produced by first liquefying starch to DE 10-20 and then adjusting the pH and

temperature to 55°C and a pH around 5.5, respectively, and  
subjecting the liquefied starch to a fungal  $\alpha$ -amylase activity  
(e.g., Fungamyl™ 800L) for 22-44 hours. The dosage of fungal  $\alpha$ -  
amylase depends on the saccharification time foreseen, e.g., 200  
s g/t DS for 44 hours and 400 g/t DS for 22 hours.

To produce High Maltose Syrup starch with maltose content of 55-  
65% starch is liquefied to DE 10-20. The pH and temperature of  
the liquefied starch is adjusted to 60°C and to a pH around 6,  
10 respectively, and is subjected to maltogenic amylase variant  
activity (e.g., 4000 MANU/g, 0.25-1.0 l/t DS), and fungal  $\alpha$ -  
amylase activity (e.g., Fungamyl™ 800 L, 0.4-1.0 kg/t DS) for 24-  
48 hours.

15 Alternatively, the liquefied starch may adjusted to a temperature  
of 65°C and a pH around 5.0 and subjected to maltogenic amylase  
activity (e.g., 4000 MANU/g, 0.5-1.0 l/t DS), and pullulanase  
activity (e.g., Promozyme™ 600 L, 0.5-1.0 l/t DS) for 18-42  
hours.

20

Production of Ultra High Maltose Syrup (containing above 80% maltose)

To produce a Ultra High Maltose Syrup starch is liquefied to a  
DE of max. 10, DS=30%. The pH and temperature of the liquefied  
25 starch is adjusted 58°C and a pH around 5.5, respectively, and  
is subjected to a maltogenic amylase activity (e.g., 4000 MANU/g,  
1.5 l/t DS), pullulanase activity (e.g., Promozyme™ 600 L, 1 l/t  
DS) and malt extract (1500° Lintner) 1 kg/t DS, for a period of  
time from 24-72 hours. The specific process time depends on the  
30 desired saccharide spectrum to be achieved.

**Speciality Syrup production using Maltogenic amylase variants**

The invention also relates to producing Speciality Syrups. The  
term "Speciality Syrups", is an art recognised term and is  
35 characterised according to DE and carbohydrate spectrum (See the  
article "New Speciality Glucose Syrups", p. 50+, in the  
textbook "Molecular Structure and Function of Food

Carbohydrate'', Edited by G.G. Birch and L.F. Green, Applied Science Publishers LTD., London). Typically Speciality Syrups have a DE in the range from 35 to 45.

- 5 Speciality Syrups are used in the brewing industry as a supplement to the beer wort (adjuncts). The main purpose for adding these syrups is to provide fermentable sugars to the yeast, i.e., glucose, maltose and maltotriose. The presence of higher oligosaccharides is, however, also important in supplying  
10 body and flavour to the beer. Due to different traditions in the brewing industry the use of adjuncts is not accepted all over the world, i.e., Europe and Japan still favour old fashion brewing practices. Furthermore, the desired carbohydrate spectrum of the adjunct syrup is highly variable. Whereas, some brewers want a  
15 syrup with equal amounts of glucose and maltose, others prefer one with very little glucose, i.e., high maltose syrups.

- In an aspect of the invention an immobilised maltogenic amylase variant may advantageously be used for the production of  
20 Speciality Syrups, e.g., for brewing. The Speciality Syrup can be produced by adding, e.g., an about 40 DE enzyme liquefied starch to an immobilised maltogenic amylase enzyme column. The starch substrate may be made by liquefying starch with an  $\alpha$ -amylase at 95°C/1 hour followed by 80°C/24-48 hours to a final DE of  
25 approximately 40.

The maltogenic amylase variants may be immobilised using any method known in the art. One method is described in the following:

- 30 The semi-purified maltogenic amylase enzymes are immobilised by crosslinking with glutaraldehyde, polyethyleneimine (PEI) and albumin (extra inert protein). 15 g albumin is stirred with 150 g deionised water and 250 g enzyme solution is added (8 g of enzyme in water or filtered fermentation broth). pH is adjusted to 6.5.  
35 8% glutardialdehyde is added (w/w on DS of enzyme + albumin, using a 50% glutardialdehyde solution), followed by 10% PEI (w/w) on DS (enzyme + albumin, 30% PEI solution) and 16% glutardialdehyde (w/w) on DS (enzyme + albumin). All additions

are made slowly under heavy stirring and constant pH adjustment at 6.5. The mixture is left without stirring for 60 minutes. 15-30 ml, 0.1% Superfloc A 130 is added under gentle stirring and the mixture left for 10 minutes. After flocculation the mixture is filtered through 6 sheets of gaze, wrung and the dry mass extruded through a sieve. The granulate is dried at room temperature and sieved. The fraction 300-1000  $\mu\text{m}$  is used. Yields around 20 g granulate are obtained.

Finally, the invention comprises the use of the limit dextrin of the invention as a fat replacer in foods. It has been found that this very cheap limit dextrin can be used as a fat replacer, which exhibits the same good organoleptic properties as traditional fat replacers. Also, the limit dextrin according to the invention can be used in confections with a gum structure, in soft drinks, in viscous dairy products, and as a carrier for dried liquids as well as a paper sizing agent.

#### Maltogenic amylases

As indicated above, a maltogenic amylase (glucan 1,4- $\alpha$ -maltohydrolase, E.C. 3.2.1.133) is able to hydrolyse amylose and amylopectin to maltose in the  $\alpha$ -configuration. Furthermore, a maltogenic amylase is able to hydrolyse maltotriose as well as cyclodextrin.

A maltogenic amylase from *Bacillus* (EP 120 693) is commercially available from Novo Nordisk A/S, Denmark and is widely used in the starch industry. It is most active at 60-70°C (Christophersen, C., et al., 1997, Starch, vol. 50, No. 1, 39-45). This particular maltogenic amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1.

Also, this particular maltogenic amylase shares several characteristics with cyclodextrin glucanotransferases (CGTases), including sequence homology (Henrissat B., Bairoch A. 1996) and formation of transglycosylation products (Christophersen, C., et al., 1997, Starch, vol. 50, No. 1, 39-45). Cyclomaltodextrin

glucanotransferase (E.C. 2.4.1.19), also designated cyclodextrin glucanotransferase or cyclodextrin glycosyltransferase, abbreviated herein as CGTase, catalyses the conversion of starch and similar substrates into cyclomaltodextrins via an intramolecular transglycosylation reaction, thereby forming cyclomaltodextrins (or CD) of various sizes.

As mentioned above, maltogenic amylase is an enzyme classified in EC 3.2.1.133. The enzymatic activity does not require a non-reducing end on the substrate and the primary enzymatic activity results in the degradation of amylopectin and amylose to maltose and longer maltodextrins. It is able to hydrolyze amylose and amylopectin to maltose in the alpha-configuration, and is also able to hydrolyze maltotriose as well as cyclodextrin.

One interesting maltogenic amylase from which variants can be prepared is the amylase cloned from *Bacillus* as described in EP 120 693, which has the amino acid sequence set forth in amino acids 1-686 of SEQ ID NO:1. This maltogenic amylase is encoded in the gene harbored in the *Bacillus* strain NCIB 11837 which has the nucleic acid sequence set forth in SEQ ID NO:1. The three-dimensional structure of the above-mentioned maltogenic amylase is described below.

In general, a preferred maltogenic amylase should have one or more of the following properties:

- i) a three dimensional structural homology to the maltogenic amylase shown as amino acids 1-686 of SEQ ID NO:1,
- ii) maltogenic amylase activity,
- iii) an amino acid sequence having at least 70% identity to amino acids 1-686 of SEQ ID NO:1, preferably at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%,
- iv) optimum maltogenic activity in the range pH 3.5-7.0, preferably in the range pH 4-5.5.

### Three-dimensional structure of maltogenic amylase

The structure of maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 is made up of five globular domains, ordered A, B, C, D and E. The domains can be defined as being residues 1-132 and 204-403 for Domain A, residues 133-203 for Domain B, residues 404-496 for Domain C, residues 497-579 for Domain D, and residues 580-686 for Domain E, wherein the numbering refers to the amino acid sequence in SEQ ID NO: 1. Features of Domains A, B, and C of particular interest are described below.

#### Domain A

Domain A is the largest domain and contains the active site which comprises a cluster of three amino acid residues, D329, D228 and E256, spatially arranged at the bottom of a cleft in the surface of the enzyme. The structure of Domain A shows an overall fold in common with the  $\alpha$ -amylases for which the structure is known, viz. the (beta/alpha) 8 barrel with eight central beta strands (numbered 1-8) and eight flanking  $\alpha$ -helices. The  $\beta$ -barrel is defined by McGregor *op. cit.* The C-terminal end of the beta strand 1 is connected to helix 1 by a loop denoted loop 1 and an identical pattern is found for the other loops, although the loops show some variation in size and some can be quite extensive.

The eight central beta-strands in the (beta/alpha) 8 barrel superimpose reasonably well with the known structures of CGTases. This part of the structure, including the close surroundings of the active site located at the C-terminal end of the beta-strands, shows a high degree of identity with CGTases.

In contrast, the loops connecting the beta-strands and alpha helices display a high degree of variation from the known structures of CGTases. These loops constitute the structural context of the active site, and the majority of the contacts to the substrate is found among residues located in these loops. Distinguishing characteristics such as substrate specificity, substrate binding, pH activity profile, substrate cleavage

pattern, and the like, are determined by specific amino acids and the positions they occupy in these loops. In the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1, Domain A contains two calcium binding sites, one of which is homologous to the calcium binding site in CGTases; the other is unique to the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1. The structure of the calcium binding site is discussed further below in the section "Calcium binding sites."

#### 10 Domain E

Domain E, also referred to as loop 3 of the (beta/alpha) 8 barrel, in comprises amino acid residues 133-203 of the amino acid sequence shown in SEQ ID NO: 1. The structure is partially homologous to the structure of Domain E in CGTases, the most striking difference being the presence of a five amino acid insert corresponding to positions 191-195 in the amino acid sequence shown in SEQ ID NO: 1 which is not found in the CGTases. This insert is spatially positioned close to the active site residues and in close contact to the substrate.

#### 20 Domain C

Domain C in the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 comprises amino acid residues 404-496 of the amino acid sequence shown in SEQ ID NO: 1. Domain C is composed entirely of  $\beta$ -strands which form a single 8-stranded sheet structure that folds back on itself, and thus may be described as a  $\beta$ -sandwich structure. One part of the  $\beta$ -sheet forms the interface to Domain A.

#### Calcium binding sites

The structure of the maltogenic amylase exhibits three calcium-binding sites; that is, three calcium ions are found to be present in the structure. In common with most of the known family 13 structures, one calcium ion is located between the A and B domains. This calcium ion is coordinated by a backbone carbonyl atom from Gln184 and His232, sidechain atoms from Asp198, a sidechain atom from Asn131, and three water molecules W8.

A second calcium ion is located in the A domain and is common to CGTases, but not found in  $\alpha$ -amylases. The calcium ion is coordinated by a backbone carbonyl atom from Gly48 and Asp23, a  
5 sidechain atom from Asp50, a sidechain atom from Asp21, a sidechain atom from Asn26, and a sidechain atom from Asn27, and one water molecule.

The third calcium ion is located in the A Domain and is unique to the maltogenic amylase having the amino acid sequence shown as  
10 amino acids 1-686 of SEQ ID NO:1. The coordination comprises a backbone carbonyl atom from Asn77, sidechain atoms from Glu102, a sidechain atom from Asp79, a sidechain atom from Asp76, a sidechain atom from Glu101, and one water molecule.

#### Substrate Binding Site

15 Parts of the loop discussed above in the context of domains A and B are of particular interest for substrate interaction and active site reactivity. In particular, in domain A, residues 37-45 in loop 1, residues 261-266 in loop 5, residues 327-330 in loop 7 and residues 370-376 in loop 8; in domain B, residues 135-145 in  
20 loop 3, residues 173-180 and 188-196 in loop 3, wherein residue positions correspond to the amino acids in the amino acid sequence in SEQ ID NO: 1.

Without being limited to any theory, it is presently believed  
25 that binding between a substrate and an enzyme is supported by favorable interactions found within a sphere of 4 to 6 Å between the substrate molecule and the enzyme, such as hydrogen bonds and/or strong electrostatic interaction. The following residues of the maltogenic amylase (SEQ ID NO: 1), are within a distance  
30 of 6 Å of the substrate HEX and thus believed to be involved in interactions with said substrate:

44, 89, 90, 92, 93, 127, 129, 132, 135, 177, 178, 188, 191, 194, 196, 226, 228, 229, 230, 231, 232, 256, 258-261, 288, 328, 329, 371, 372, 373, 376, and 690.

35

The following residues of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 are

within a distance of 4 Å of the substrate HEX and thus believed to be involved in interactions with said substrate:

90, 92, 93, 129, 132, 177, 188, 189, 190, 191, 196, 226, 228, 229, 231, 232, 256, 258, 259, 260, 261, 328, 329, 372, 376, and  
5 690.

#### Homology building of maltogenic amylases

A model structure of a maltogenic amylase can be built using the Homology program or a comparable program, e.g., Modeller (both  
10 from Molecular Simulations, Inc., San Diego, CA). The principle is to align the sequence of the maltogenic amylase with the known structure with that of the maltogenic amylase for which a model structure is to be constructed. The structurally conserved regions can then be built on the basis of consensus sequences. In  
15 areas lacking homology, loop structures can be inserted, or sequences can be deleted with subsequent bonding of the necessary residues using, e.g., the program Homology. Subsequent relaxing and optimization of the structure should be done using either Homology or another molecular simulation program, e.g., CHARMM  
20 from Molecular Simulations.

#### Maltogenic amylase variants with altered thermostability and/or altered temperature dependent activity profile

As explained above, one of the advantages of using variants of the maltogenic amylase having the amino acid sequence shown as  
25 amino acids 1-686 of SEQ ID NO:1 in the starch processing procedure is the possibility of carrying out the reaction at elevated temperatures. Accordingly, variants which are particularly interesting for the purposes described herein are such variants which

30

- a) has maltogenic amylase activity,
- b) has at least 70% identity to amino acids 1-686 of SEQ ID NO: 1,
- c) has optimum maltogenic amylase activity in the range pH 3.5-  
35 7.0, and
- d) show a residual maltogenic amylase activity of at least 25% after incubation with 1 mM Ca<sup>++</sup> at pH 4.3, 80°C for 15 minutes.

- Preferably, the variant should possess as high a thermostability as possible and, accordingly, variants which are particular preferred are variants which fulfill the criteria set forth
- 5 under d) above at an even higher level, e.g. variants which show a residual maltogenic amylase activity of at least 30%, e.g. at least 40%, preferably at least 50%, e.g. at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90% after incubation with 1 mM  $\text{Ca}^{++}$  at pH 4.3, 80°C for 15 minutes.
- 10 The structure of the maltogenic amylase contains a number of unique internal cavities which may contain water and a number of crevices. In order to increase the thermostability of the variant it may be desirable to reduce the number or size of cavities and crevices, e.g., by introducing one or more hydrophobic contacts,
- 15 preferably achieved by introducing amino acids with bulkier side groups in the vicinity or surroundings of the cavity. For instance, the amino acid residues to be modified are those which are involved in the formation of the cavity.
- 20 It will be understood that the cavity or crevice is identified by the amino acid residues surrounding said cavity or crevice, and that modification of said amino acid residues are of importance for filling or reducing the size of said cavity or crevice. Preferably, the modification is a substitution with a bulkier
- 25 amino acid residue, i.e. one with a greater side chain volume. For example, all the amino acids are bulkier than Gly, whereas Tyr and Trp are bulkier than Phe. The particular amino acid residues referred to below are those that in a crystal structure have been found to flank the cavity or crevice in question.
- 30 Thus, in a preferred embodiment the maltogenic amylase to be used for producing the enzymatically modified starch comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:
- 35

L51, L71, S72, V74, L75, L76, T80, L81, G83, T84, D85, N86, T87, G88, Y89, H90, G91, T94, R95, D96, F97, V114, I125, V126, T134,

G157, Y167, F168, H169, H170, N171, G172, D173, I174, S175, N176, D178, D179, R180, Y181, E182, A183, Q184, K186, N187, F188, T189, D190, A192, G193, F194, S195, L196, L217, S235, G236, V254, V279, V281, L286, V289, I290, V308, L321, I325, 5 D326, L343, F349, S353, I359, I405, L448, Q449, L452, I470, G509, V515, S583, G625, L627, L628 or A670.

In a more preferred embodiment, the variant of a maltogenic amylase comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in 10 SEQ ID NO: 1:

L217 in combination with L75 (e.g. L217F/Y in combination with L75F/Y), L51W, L75F/Y, L78I, G88A/V/T, G91T/S/V/N, T94V/I/L, V114V/I/L, I125L/M/F/Y/W, V126I/L, T134V/I/L/M/F/Y/W, 15 G157A/V/I/L, L217V/I/M/F/Y/W, S235I/L/M/F/Y/W, G236A/V/I/L/M/F/Y/W, V254I/L/M/F/Y/W, V279M/I/L/F, V281I/L/M/F/Y/W, L286F, V289I/L/R, I290M/L/F, V308I/L/M/F/Y/W, L321I/M/F/Y/W, I325L/M/F/Y/W, D326E/Q, L343M/F/Y/W, F349W/Y, S353V/I/L, I359L/M/F/Y/W, I405M/L/Y/F/W, L448Y, Q449Y, 20 L452M/Y/F/W, I470M/L/F, G509A/V/I/L/M/S/T/D/N, V515I/L, S583V/I/L/V, G625A/V/I/L/M/F/Y/W, L627M/F/Y, L628M/I/F/Y/W, A670V/I/L/M/F/Y/W, L71I, S72C, V74I, L75N/D/Q/I/V, L78N/I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, G83A/S/T/N/Q/E/D/R/H/L, T84S/A/N/D/G, D85A/T/S/N/G, N86Q/E/D/Y/H/K, T87S/I, G88A/S/T, 25 Y89F, H90N/Q/K, G91A/S/T, T94N/D/A/M/V/I, R95K/Q, D96N/V/Q/I, F97Y, Y167F/R/C, F168Y, H169N/Q/K, H170N/Q/K, N171D/E/Q/H/R/K/G, G172A/T/S, D173N/S/T/Y/R/G, I174N/Q/L, S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W, Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R, N187Q/E/L/F/H/K/V/L, 30 F188Y/L/I/H/N, T189N/D/A/S/H/Y/G, D190E/Q/H/N/K, A192T/D/E/N/K, G193A/S/T, F194Y, S195N/D/E/R/K/G, and L196I.

Similar substitutions may be introduced in equivalent positions of other maltogenic amylases. Variants of particular interest 35 have a combination of one or more of the above with any of the other modifications disclosed herein.

**Maltogenic amylase variants with altered stability**

In general, variants having improved (increased) stability may be obtained by stabilization of calcium binding, substitution with proline, substitution of histidine with another amino acid, introduction of an interdomain disulfide bond, removal of a deamidation site, altering a hydrogen bond contact, filling in an internal structural cavity with one or more amino acids with bulkier side groups, introduction of interdomain interactions, altering charge distribution, helix capping, or introduction of a salt bridge.

Calcium binding

Variants which are contemplated to be suitable for the starch processing procedure described herein are variants which have an altered stability due to an altered stabilization of calcium ( $\text{Ca}^{2+}$ ) binding. The enzyme variant may have altered thermostability or pH dependent stability, or it may have maltogenic amylase activity in the presence of a lower concentration of calcium ion. It is presently believed that amino acid residues located within 10 Å from a calcium ion are involved in or are of importance for the  $\text{Ca}^{2+}$  binding capability of the enzyme.

The amino acid residues found within a distance of 10 Å from the  $\text{Ca}^{2+}$  binding sites of the maltogenic amylase with the amino acid sequence set forth in SEQ ID NO: 1 are as follows:

16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 35, 36, 40, 46, 47, 48, 49, 50, 51, 52, 53, 54, 56, 73, 74, 75, 76, 77, 78, 79, 80, 81, 87, 88, 89, 91, 93, 94, 95, 96, 99, 100, 101, 102, 103, 104, 105, 109, 129, 130, 131, 132, 133, 134, 145, 150, 167, 168, 169, 170, 171, 172, 174, 177, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 196, 197, 198, 199, 200, 201, 202, 206, 210, 228, 229, 230, 231, 232, 233, 234, 235, 237, 378, and 637.

35

In order to construct a variant, it is desirable to substitute at least one of the above mentioned amino acid residues, which is

determined to be involved in a non-optimal calcium binding, with any other amino acid residue which improves the  $\text{Ca}^{2+}$  binding affinity of the variant enzyme. Accordingly, one method of constructing a variant of a parent maltogenic amylase wherein  
5 said variant has a stabilised  $\text{Ca}^{2+}$  binding as compared to said parent amylase comprises:

i) identifying an amino acid residue within 10 Å from a  $\text{Ca}^{2+}$  binding site of a maltogenic amylase in a model of the three-dimensional structure of said amylase which, from structural or  
10 functional considerations, is determined to be responsible for a non-optimal calcium ion interaction;

ii) constructing a variant in which said amino acid residue is substituted with another amino acid residue which, from  
15 structural or functional considerations, is determined to be important for establishing an altered  $\text{Ca}^{2+}$  binding affinity; and

iii) testing the  $\text{Ca}^{2+}$  binding of the resulting maltogenic amylase variant.

20

Substituting an amino acid residue responsible for non-optimal calcium ion interaction with another residue may alter a calcium ion binding interaction of the enzyme. For instance, the amino acid residue in question may be selected on the basis of one or  
25 more of the following objectives:

a) to obtain an improved interaction between a calcium ion and an amino acid residue as identified from the structure of the maltogenic amylase. For instance, if the amino acid residue in  
30 question is exposed to a surrounding solvent, it may be advantageous to increase the shielding of said amino acid residue from the solvent so as to stabilize the interaction between said amino acid residue and a calcium ion. This can be achieved by substituting said residue, or an amino acid residue in the  
35 vicinity of said residue contributing to the shielding, with an amino acid residue with a bulkier side group or which otherwise results in an improved shielding effect.

b) to stabilize a calcium binding site, for instance by stabilizing the structure of the maltogenic amylase, e.g. by stabilizing the contacts between two or more of the five domains or stabilizing one or more of the individual domains as such.

5 This may, e.g., be achieved by providing for a better coordination to amino acid side chains, which may, e.g., be obtained by substituting an N residue with a D residue and/or a Q residue with an E residue, e.g. within 10 Å, and preferably within 3 or 4 Å, of a calcium binding site.

10

c) to improve the coordination between the calcium ion and the calcium binding residues, e.g., by improving the interaction between the ion and the coordinating residues or increasing the number of sidechain coordinations by substituting a coordinating  
15 water with an amino acid sidechain.

d) replace water by a coordinating calcium amino acid residue.

Preferably, the amino acid residue to be modified is located  
20 within 8 Å of a  $\text{Ca}^{2+}$  ion, in particular within 5 Å of a  $\text{Ca}^{2+}$  ion.

In a preferred embodiment, the variant of a maltogenic amylase having an altered  $\text{Ca}^{2+}$  binding as compared to the parent maltogenic amylase comprises a substitution of an amino acid  
25 residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

D17, A30, S32, R95, H103, N131, Q201, I174, H169, V74, L75, L78,  
T80, L81, T87, G88, Y89, H90, G91, T94, R95, D96, F97, Y167,  
30 F169, H169, H170, N171, G172, D173, I174, S175, N176, D178, D179,  
R180, Y181, E182, A183, Q184, K186, N187, F188, and/or T189.

In more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to one or more of the  
35 following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

D17E/Q, A30M/L/A/V/I/E/Q, S32D/E/N/Q, R95M/L/A/V/I/E/Q,  
H103Y/N/Q/D/E, N131D, Q201E, I174E/Q, H169N/D/E/Q, V74I,  
L75N/D/Q/I/V, L78N/I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, T87S/I,  
G88A/S/T, Y89F, H90N/Q/K, G91A/S/T, T94N/D/A/M/V/I, R95K/Q,  
5 D96N/V/Q/I, F97Y, Y167F/R/C, F168Y, H169N/Q/K, H170N/Q/K,  
N171D/E/Q/H/R/K/G, G172A/T/S, D173N/S/T/Y/R/G, I174N/Q/L,  
S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W,  
Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R, N187Q/E/L/F/H/K/V/L,  
F188Y/L/I/H/N, and/or T189N/D/A/S/H/Y/G.

10

In another preferred embodiment of the invention with respect to altering the  $\text{Ca}^{2+}$  binding of a maltogenic amylase the partial sequence N28-P29-A30-K31-S32-Y33-G34 as set forth in SEQ ID NO: 1 is modified.

15

Similar substitutions may be introduced in equivalent positions of other maltogenic amylases. Modifications of particular interest are any combination of one or more of the above with any of the other modifications disclosed herein.

#### 20 Other substitutions

Variants with improved stability of the enzyme can be achieved by improving existing or introducing new interdomain and intradomain contacts. Such improved stability can be achieved by the modifications listed below.

25

The maltogenic amylase having the amino acid sequence shown in SEQ ID NO: 1 may be stabilized by the introduction of one or more interdomain disulfide bonds. Accordingly, another preferred embodiment of the present invention relates to a variant of a  
30 parent maltogenic amylase which has improved stability and at least one more interdomain disulfide bridge as compared to said parent, wherein said variant comprises a modification in a position corresponding to at least one of the following pairs of positions in SEQ ID NO: 1:

35

G236 + S583, G618 + R272, T252 + V433 and/or A348 + V487.

In a more preferred embodiment, the substitution corresponds to at least one of the following pairs:

G236C + S583C, G618C + R272C, T252C + V433C and/or A348C + V487C.

5 In another interesting embodiment of the invention variants of a parent maltogenic amylase which have an improved stability and an altered interdomain interaction as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position  
10 corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

i) F143, F194, L78;

ii) A341, A348, L398, I415, T439, L464, L465;

15 iii) L557;

iv) S240, L268;

v) Q208, L628;

vi) F427, Q500, N507, M508, S573; and/or

vii) I510, V620.

20

In a more preferred embodiment, the substitution corresponds to at least one of the following sets:

i) F143Y, F194Y, L78Y/F/W/E/Q;

ii) A341S/D/N, A348V/I/L, L398E/Q/N/D, I415E/Q, T439D/E/Q/N,

25 L464D/E, L465D/E/N/Q/R/K;

iii) L557Q/E/N/D;

iv) S240D/E/N/Q, L268D/E/N/Q/R/K;

v) Q208D/E/Q, L628E/Q/N/D;

vi) F427E/Q/R/K/Y, Q500Y, N507Q/E/D, M508K/R/E/Q, S573D/E/N/Q;

30 and/or

vii) I510D/E/N/Q/S, V620D/E/N/Q.

In a further interesting embodiment of the invention variants of a parent maltogenic amylase which have an improved stability and  
35 one or more salt bridges as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position

corresponding to at least one of the following sets of positions in SEQ ID NO: 1: N106, N320 and Q624.

In a more preferred embodiment, the variant of a maltogenic  
5 amylase comprises a substitution corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1: N106R, N320E/D and/or Q624E.

In a still further interesting embodiment variants of a parent  
10 maltogenic amylase which have an improved stability as compared to said parent enzyme may be used for the purposes described herein. Examples of such a variants include variants comprising a substitution in a position corresponding to at least one of the following sets of positions in SEQ ID NO: 1:

15 K40, V74, S141, T142, F188, N234, K249, D261, L268, V279, N342, G397, A403, K425, S442, S479, S493, T494, S495, A496, S497, A498, Q500, K520, A555 and N595.

20 In a more preferred embodiment, the variant of a maltogenic amylase comprises a substitution corresponding to one or more of the following substitutions with proline in the amino acid sequence set forth in SEQ ID NO: 1:

25 V74P, S141P, N234P, K249P, L268P, V279P, N342P, G397P, A403P, S442P, S479P, S493P, T494P, S495P, A496P, S497P, A498P, Q500P, and/or A555P.

Other preferred substitutions are K40R, T142A, F188I/L, D261G,  
30 K425E, K520R, and/or N595I.

Analogously, it may be preferred that one or more histidine residues present in the parent maltogenic amylase is or are substituted with a non-histidine residues such as Y, V I, L, F,  
35 M, E, Q, N, or D. Accordingly, in another preferred embodiment, the variant of a maltogenic amylase comprises a substitution of an amino acid residue corresponding to one or more of the

following residues of the amino acid sequence set forth in SEQ ID NO: 1: H103, H220, and H344

In a more preferred embodiment, the variant of a maltogenic  
5 amylase comprises a substitution corresponding to one or more of the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1: H103Y/V/I/L/F/Y, H220Y/L/M, and H344E/Q/N/D/Y.

It may be preferred that one or more asparagine or glutamine  
10 residues present in the parent maltogenic amylase is or are substituted with a residue lacking the amide on the side chain. Accordingly, in another preferred embodiment, the variant of a maltogenic amylase-like enzyme comprises a substitution of an amino acid residue corresponding to one or more of the following  
15 residues of the amino acid sequence set forth in SEQ ID NO: 1:

Q13, N26, N77, N86, N99, Q119, N120, N131, N152, N171, N176,  
N187, Q201, N203, N234, Q247, N266, N275, N276, N280, N287, Q299,  
N320, N327, N342, Q365, N371, N375, N401, N436, N454, N468, N474,  
20 Q500, N507, N513, Q526, N575, Q581, N621, Q624 and N664.

In a more preferred embodiment, the variant of a maltogenic  
amylase comprises a substitution corresponding to one or more of the following substitutions in the amino acid sequence set forth  
25 in SEQ ID NO: 1:

Q13S/T/A/V/L/I/F/M, N26S/T/A/V/L/I, N77S/T/A/V/L/I,  
N86S/T/A/V/L/I, N99T/S/V/L, Q119T/S, N120S/T/A/V/L/I,  
N131S/T/A/V/L/I, N152T/S/V/L, N171Y/D/S/T, N176S/T/A/V/L/I,  
30 N187S/T/A/V/L/I, Q201S/T/A/V/L/I/F/M, N203D/S/T/A/V/L/I,  
N234S/T/A/V/L/I, Q247S/T/A/V/L/I/F/M, N266S/T/A/V/L/I,  
N275S/T/A/V/L/I, N276S/T/A/V/L/I, N280S/T/A/V/L/I,  
N287S/T/A/V/L/I, Q299L/T/S, N320S/T/A/V/L/I, N327S/T/A/V/L/I,  
N342S/T/A/V/L/I, Q365S/T/A/V/L/I, N371S/T/A/V/L/I,  
35 N375S/T/A/V/L/I, N401S/T/A/V/L/I, N436S/T/A/V/L/I,  
N454D/S/T/A/V/L/I, N468D/S/T/A/V/L/I, N474D/S/T/A/V/L/I,  
Q500S/T/A/V/L/I/F/M, N507S/T/A/V/L/I, N513S/T/A/V/L/I, Q526

D/S/T/A/V/L/I, N575S/T/A/V/L/I, Q581S/T/A/V/L/I/F/M,  
N621S/T/A/V/L/I Q624S/T/A/V/L/I/F/M and N664D/S/T/A/V/L/I.

In a further interesting embodiment of the invention variants of  
5 a parent maltogenic amylase which have an improved stability and  
improved hydrogen bond contacts as compared to said parent enzyme  
may be used for the purposes described herein. Examples of such a  
variants include variants comprising a substitution in a position  
corresponding to at least one of the following sets of positions  
10 in SEQ ID NO: 1:

I16, L35, M45, P73, D76, D79, A192, I100, A148, A163+G172, L268,  
V281, D285, L321, F297, N305, K316, S573, A341, M378, A381, F389,  
A483, A486, I510, A564, F586, K589, F636, K645, A629, and/or  
15 T681.

In a preferred embodiment, the modification corresponds to one or  
more of the following:

20 I16T/D/N, L35Q, M45K, P73Q, D76E, D79E/Y, A192S/D/N,  
I100T/S/D/N/E/Q, A148D/N/E/Q/S/T/R/K, A163Y+G172S/D/N, L268R/K,  
V281/Q, D285R/K, L321Q, F297N/D/Q/E, N305K/R, K316N/D, S573N/D,  
A341R/K, M378R/K, A381S/D/N, F389Y, A483S/D/N, A486Q/E, I510R/K,  
A564S/D/N, F586S/D/N, K589S/D/Q/N, F636Y, K645T, A629N/D/E/Q,  
25 and/or T681D/N/E/Q/S.

Similar substitutions may be introduced in equivalent positions  
of other maltogenic amylases. Substitutions of particular  
interest are any combination of one or more of the above with any  
30 of the other modifications disclosed herein.

Before actually constructing a maltogenic amylase variant to  
achieve any of the above objectives, it may be convenient to  
evaluate whether or not the contemplated amino acid modification  
35 can be accommodated into the maltogenic amylase structure, e.g.  
into a model of the three-dimensional structure of the parent  
maltogenic amylase.

**Maltogenic amylase variants with altered pH dependent activity profile**

Variants having an altered pH dependent activity profile may also be suitable for the purposes described herein. The pH dependent activity profile can be changed by changing the pKa of residues within 10 Å of the active site residues of the maltogenic amylase. Changing the pKa of the active site residues is achieved, e.g., by changing the electrostatic interaction or hydrophobic interaction between functional groups of amino acid side chains of a given amino acid residue and its close surroundings. To obtain a higher activity at a higher pH, negatively charged residues are placed near a hydrogen donor acid, whereas positively charged residues placed near a nucleophilic acid will result in higher activity at low pH. Also, a decrease in the pKa can be obtained by reducing the accessibility of water or increasing hydrophobicity of the environment.

Thus, a variant having an altered pH dependent activity profile as compared to the parent enzyme, may be obtained by the following method:

- i) identifying an amino acid residue within 15 Å from an active site residue of a maltogenic amylase in the three-dimensional structure of said parent maltogenic amylase, in particular 10 Å from an active site residue, wherein said amino acid residue is contemplated to be involved in electrostatic or hydrophobic interactions with an active site residue;
- ii) substituting, in the structure, said amino acid residue with an amino acid residue which changes the electrostatic and/or hydrophobic surroundings of an active site residue, and evaluating the accommodation of the amino acid residue in the structure,
- iii) optionally repeating step i) and/or ii) recursively until an amino acid substitution has been identified which is accommodated into the structure,

iv) constructing a maltogenic amylase variant resulting from steps i) and ii), and optionally iii), and testing the pH dependent enzymatic activity of said variant.

- 5 In a preferred embodiment, the variant of a maltogenic amylase having an altered pH dependent activity profile as compared to the parent maltogenic amylase comprises a modification of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:

10

D127, V129, F188, A229, Y258, V281, F284, T288, N327, M330, G370, N371, D372, L71, S72, V74, L75, L78, T80, L81, G83, T84, D85, N86, T87, G88, Y89, H90, G91, T94, R95, D96, F97, I174, S175, N176, D178, D179, R180, Y181, E182, A183, Q184, K186, N187, F188, 15 T189, D190, A192, G193, F194, S195, and L196.

In more preferred embodiment, the variant comprises a modification corresponding to one or more of the following modifications in the amino acid sequence set forth in SEQ ID NO:

- 20 1: D127N/L, V129S/T/G/V, F188E/K/H, A229S/T/G/V, Y258E/D/K/R/F/N, V281L/T, F284K/H/D/E/Y, T288E/K/R, N327D, M330L/F/I/D/E/K, G370N, N371D/E/G/K, D372N/V, L71I, S72C, V74I, L75N/D/Q/I/V, L78N/I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, G83A/S/T/N/Q/E/D/R/H/L, T84S/A/N/D/G, D85A/T/S/N/G, N86Q/E/D/Y/H/K, T87S/I, G88A/S/T, 25 Y89F, H90N/Q/K, G91A/S/T, T94N/D/A/M/V/I, R95K/Q, D96N/V/Q/I, F97Y, I174N/Q/L, S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W, Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R, N187Q/E/L/F/H/K/V/L, F188Y/L/I/H/N, T189N/D/A/S/H/Y/G, D190E/Q/H/N/K, A192T/D/E/N/K, G193A/S/T, F194Y, S195N/D/E/R/K/G, 30 and L196I.

- Similar modifications may be introduced in equivalent positions of other maltogenic amylases. Variants of particular interest have a combination of one or more of the above with any of the 35 other modifications disclosed herein.

**Maltogenic amylase variants with an altered cleavage pattern**

For the purposes of preparing the enzymatically modified starch and starch derivatives described herein it may be desirable to change cleavage pattern of the applied enzyme, i.e. it may be desirable to change the cleavage pattern, for example, so as to form higher amounts of higher oligosaccharides.

A variant of a parent maltogenic amylase in which the substrate cleavage pattern is altered as compared to said parent may be constructed by a method which comprises:

i) identifying the substrate binding area of the parent maltogenic amylase in a model of the three-dimensional structure, e.g. within a sphere of 4 Å from the substrate binding site as defined in the section above entitled "Substrate Binding Site";

ii) substituting in the model one or more amino acid residues of the substrate binding area of the cleft identified in i) which is or are believed to be responsible for the cleavage pattern of the parent with another amino acid residue which from structural or functional considerations is believed to result in an altered substrate cleavage pattern, or deleting one or more amino acid residues of the substrate binding area contemplated to introduce favorable interactions to the substrate or adding one or more amino acid residues to the substrate binding area contemplated to introduce favorable interactions to the substrate; and

iii) constructing a maltogenic amylase variant resulting from step ii) and testing the substrate cleavage pattern of the variant.

Thus, examples of variants having an altered cleavage pattern which are considered to be useful for the purposes described herein are variants comprising a modification in a position corresponding to one or both of the following positions in SEQ ID NO: 1: V281 and/or A629.

In a preferred embodiment, the variant comprises a modification corresponding to: V281Q and/or A629N/D/E/Q.

Similar modifications may be introduced in equivalent positions of other maltogenic amylases. Substitutions of particular interest are any combination of one or both of the above with any of the other modifications disclosed herein.

**Maltogenic amylase variants with improved ability to reduce retrogradation of starch**

Interesting variants also include variants having improved ability to reduce the retrogradation of starch. Preferred variants comprise a modification at one or more positions corresponding to the following amino acid residues in SEQ ID NO: 1: A30, K40, N115, T142, F188, T189, P191, A192, G193, F194, S195, D261, N327, K425, K520 and N595.

In a more preferred embodiment, the variant comprises one or more modifications corresponding to the following in SEQ ID NO: 1: A30D, K40R, N115D, T142A, F188L, T189Y,  $\Delta$  (191-195), D261G, D261G, N327S, K425E, K520R and N595I.

#### **Nomenclature for amino acid modifications**

The nomenclature used herein for defining mutations is essentially as described in WO 92/05249. Thus, F188H indicates a substitution of the amino acid F (Phe) in position 188 with the amino acid H (His). V129S/T/G/V indicates a substitution of V129 with S, T, G or V.  $\Delta$  (191-195) or  $\Delta$  (191-195) indicates a deletion of amino acids in positions 191-195. 192-A-193 indicates an insertion of A between amino acids 192 and 193.

#### **Polypeptide sequence identity**

For purposes of the present invention, the degree of identity may be suitably determined according to the method described in Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45, with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The determination may be done by means of a computer program known such as GAP provided in the GCG

program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

### Hybridization

- 5 Suitable experimental conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (sodium chloride/sodium citrate, Sambrook, et al., 1989) for 10 min, and prehybridization of the
- 10 filter in a solution of 5x SSC, 5x Denhardt's solution (Sambrook, et al., 1989), 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-
- 15 13), <sup>32</sup>P-dCTP-labeled (specific activity > 1 x 10<sup>8</sup> cpm/µg) probe for 12 hours at ca. 45°C. The filter is then washed twice for 30 minutes in 2x SSC, 0.5% SDS at least 55°C (low stringency), preferably at least 60°C (medium stringency), more preferably at least 65°C (medium/high stringency), more preferably at least
- 20 70°C (high stringency), even more preferably at least 75°C (very high stringency).

Molecules which hybridize to the oligonucleotide probe under these conditions are detected by exposure to x-ray film.

### 25 Methods of preparing variants of maltogenic amylase variants

The DNA sequence encoding a parent maltogenic amylase may be isolated from any cell or microorganism producing the maltogenic amylase in question, using various methods well known in the art, for example, from the *Bacillus* strain NCIE 11837.

30

- First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the maltogenic amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labelled
- 35 oligonucleotide probes may be synthesised and used to identify maltogenic amylase-encoding clones from a genomic library

prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known  $\alpha$ -amylase gene could be used as a probe to identify maltogenic amylase-encoding clones, using hybridization and washing  
5 conditions of lower stringency.

Another method for identifying maltogenic amylase-encoding clones involves inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming amylase negative bacteria  
10 with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for maltogenic amylase, thereby allowing clones expressing maltogenic amylase activity to be identified.

15 Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are syn-  
20 thesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA  
25 origin, prepared by ligating fragments of synthetic, genomic or cDNA origin, wherein the fragments correspond to various parts of the entire DNA sequence, in accordance with techniques well known in the art. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as  
30 described in US 4,683,202 or R.K. Saiki et al. (1988).

#### Site-directed Mutagenesis

Once a maltogenic amylase-encoding DNA sequence has been isolated, and desirable sites for modification identified, modifications may be introduced using synthetic oligonucleotides.  
35 These oligonucleotides contain nucleotide sequences flanking the desired modification sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-

stranded gap of DNA, bridging the maltogenic amylase-encoding sequence, is created in a vector carrying the maltogenic amylase gene. Then the synthetic nucleotide, bearing the desired modification, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple modifications by performing minor alterations of the cassette. However, an even greater variety of modifications can be introduced at any one time by the Morinaga method because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing modifications into a maltogenic amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves a 3-step generation of a PCR fragment containing the desired modification introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the modification may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

#### Random Mutagenesis

Random mutagenesis is suitably performed either as localised or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

The random mutagenesis of a DNA sequence encoding a parent maltogenic amylase may be conveniently performed by use of any method known in the art.

In relation to the above, one method for generating a variant of a parent maltogenic-like amylase, wherein the variant exhibits increased stability at low pH and at low calcium concentration relative to the parent, comprises:

(a) subjecting a DNA sequence encoding the parent maltogenic-like amylase to random mutagenesis,

5 (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a maltogenic-like amylase variant which has an altered property relative to the parent  
10 maltogenic-like amylase.

Step (a) of the above method of the invention is preferably performed using doped primers, as described in the working examples herein (*vide infra*).

15

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis  
20 may be performed by use of any combination of these mutagenizing agents. The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable  
25 for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed  
30 by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

35 When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or

spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the maltogenic amylase enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase  
5 and ligase as deemed appropriate.

Preferably, the doping is carried out using "constant random doping", in which the percentage of wild-type and modification in each position is predefined. Furthermore, the doping may be  
10 directed toward a preference for the introduction of certain nucleotides, and thereby a preference for the introduction of one or more specific amino acid residues. The doping may be made, e.g., so as to allow for the introduction of 90% wild type and 10% modifications in each position. An additional consideration  
15 in the choice of a doping scheme is based on genetic as well as protein-structural constraints. The doping scheme may be made by using the DOPE program which, *inter alia*, ensures that introduction of stop codons is avoided.

20 When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent maltogenic amylase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

25 A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cerevisiae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the maltogenic amylase by, e.g., transforming a plasmid  
30 containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may be subsequently transformed into the expression organism.

35 The DNA sequence to be mutagenized may be conveniently present in a genomic or cDNA library prepared from an organism expressing the parent maltogenic amylase. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a

bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenising agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harbored in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to performing the expression step b) or the screening step c). Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenising agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gram negative bacteria such as *E. coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

Localized random mutagenesis

The random mutagenesis may be advantageously localized to a part of the parent maltogenic amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

10

The localized, or region-specific, random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art. Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g., by insertion into a suitable vector, and said part may be subsequently subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

For region-specific random mutagenesis with a view to improving the stability of calcium binding of a parent maltogenic amylase, codon positions corresponding to the following amino acid residues from the amino acid sequence set forth in SEQ ID NO: 1 may appropriately be targeted:

25

Residues:Regions:

16-33, 35-36, 40: 16-40

46-54, 56: 46-56

73-81: 73-81

87-89, 91, 93-96, 99-105, 109: 87-109

30

129-134, (145, 150): 129-134

167-172, 174, 177, 180-189: 167-189

196-202, 206-210: 196-210

228-235, 237: 228-237

378

35

637

With a view to achieving improved binding of a substrate, i.e., improved binding of a carbohydrate species, such as amylose or

amylopectin, by a maltogenic amylase variant with a modified, e.g. higher, substrate specificity and/or a modified, e.g. higher, specificity with respect to cleavage, i.e. hydrolysis, of the substrate, it appears that the following codon positions in the following regions of the amino acid sequence shown in SEQ ID NO: 1, may particularly appropriately be targeted for modification by region-specific mutagenesis:

70-97, 127-143, 174-198, 226-233, 255-270, 282-292, 324-331, 370-376.

For region-specific random mutagenesis with a view to altering the substrate specificity and/or the pH dependent activity profile, the following regions of SEQ ID NO: 1 may be targeted:

70-97, 174-198.

For random mutagenesis with a view to improving the thermostability, the residues and regions described above for filling internal holes, improved Ca binding, interdomain and intradomain contacts, helix capping, proline substitution, histidine substitution etc. may be targeted. In addition, the following regions may be targeted with a view to improving the thermostability: 70-109, 167-200.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme
2. Decide on mutation sites and nonmutated sites in the selected region
3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed
4. Select structurally reasonable mutations
5. Adjust the residues selected by step 3 with regard to step 4.
6. Analyse by use of a suitable dope algorithm the nucleotide distribution.

7. If necessary, adjust the wanted residues to genetic code realism, e.g. taking into account constraints resulting from the genetic code, e.g. in order to avoid introduction of stop codons; the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted
8. Make primers
9. Perform random mutagenesis by use of the primers
10. Select resulting  $\alpha$ -amylase variants by screening for the desired improved properties.

10

Suitable dope algorithms for use in step 6 are well known in the art. One such algorithm is described by Tomandl, D. et al., 1997, Journal of Computer-Aided Molecular Design 11:29-38. Another algorithm is DOPE (Jensen, LJ, Andersen, KV, Svendsen, A, and Kretschmar, T (1998) Nucleic Acids Research 26:697-702).

15

#### **Expression of maltogenic amylase variants**

The construction of the variant of interest is accomplished by cultivating a microorganism comprising a DNA sequence encoding the variant under conditions which are conducive for producing the variant, and optionally subsequently recovering the variant from the resulting culture broth. This is described in detail further below.

20

A DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in the form of a protein or polypeptide, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

30

The recombinant expression vector carrying the DNA sequence encoding an maltogenic amylase variant may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an

45

extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a maltogenic amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens*  $\alpha$ -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes, etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the maltogenic amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of

such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus*  $\alpha$ -amylases mentioned herein comprise a pre-region permitting secretion of the expressed protease into the culture medium. If desirable, this prerregion may be replaced by a different prerregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective prerregions.

The procedures used to ligate the DNA construct of the invention encoding maltogenic amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a maltogenic amylase variant. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the

host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

10

Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus*  
15 *circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

20

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus*  
25 *niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

30

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the maltogenic amylase variant of the invention. Suitable media are available from commercial suppliers  
35 or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The maltogenic amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulfate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

#### Testing of maltogenic amylase variants

- 10 Maltogenic amylase variants produced by any of the methods described above may be tested, either prior to or after purification, for amylolytic activity in a screening assay which measures the ability of the variant to degrade starch. The screening in step 10 in the above-mentioned random mutagenesis
- 15 method of the invention may be conveniently performed by use of a filter assay based on the following procedure: A microorganism capable of expressing the mutated maltogenic amylase of interest is incubated on a suitable medium and under suitable conditions for secretion of the enzyme, the medium being covered with two
- 20 filters comprising a protein-binding filter placed under a second filter exhibiting a low protein binding capability. The microorganism is grown on the second, top filter. Subsequent to the incubation, the bottom protein-binding filter comprising enzymes secreted from the microorganism is separated from the
- 25 second filter comprising the microorganism. The protein-binding filter is then subjected to screening for the desired enzymatic activity, and the corresponding microbial colonies present on the second filter are identified. The first filter used for binding the enzymatic activity may be any protein-binding filter, e.g.,
- 30 nylon or nitrocellulose. The second filter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins, e.g., cellulose acetate or Durapore™.
- 35 Screening consists of treating the first filter to which the secreted protein is bound with a substrate that allows detection of the  $\alpha$ -amylase activity. The enzymatic activity may be detected

by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity. The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents. For example,  $\alpha$ -amylase activity can be detected by Cibacron Red labelled amylopectin, which is immobilized in agarose.  $\alpha$ -amylase activity on this substrate produces zones on the plate with reduced red color intensity.

10

To screen for variants with increased stability, the filter with bound maltogenic amylase variants can be pretreated prior to the detection step described above to inactivate variants that do not have improved stability relative to the parent maltogenic amylase. This inactivation step may consist of, but is not limited to, incubation at elevated temperatures in the presence of a buffered solution at any pH from pH 2 to 12, and/or in a buffer containing another compound known or thought to contribute to altered stability e.g., surfactants, EDTA, EGTA, wheat flour components, or any other relevant additives. Filters so treated for a specified time are then rinsed briefly in deionized water and placed on plates for activity detection as described above. The conditions are chosen such that stabilized variants show increased enzymatic activity relative to the parent after incubation on the detection media.

To screen for variants with altered thermostability, filters with bound variants are incubated in buffer at a given pH (e.g., in the range from pH 2-12) at an elevated temperature (e.g., in the range from 50°-110°C) for a time period (e.g., from 1-20 minutes) to inactivate nearly all of the parent maltogenic amylase, rinsed in water, then placed directly on a detection plate containing immobilized Cibacron Red labelled amylopectin and incubated until activity is detectable. Similarly, pH dependent stability can be screened for by adjusting the pH of the buffer in the above inactivation step such that the parent maltogenic amylase is inactivated, thereby allowing detection of only those variants with increased stability at the pH in question. To screen for

variants with increased calcium-dependent stability calcium chelators, such as ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), is added to the inactivation buffer at a concentration such that the parent maltogenic amylase is inactivated under conditions further defined, such as buffer pH, temperature or a specified length of incubation.

The variants may be suitably tested by assaying the starch-degrading activity of the variant, for instance by growing host cells transformed with a DNA sequence encoding a variant on a starch-containing agarose plate and identifying starch-degrading host cells as described above. Further testing in regard to altered properties, including specific activity, substrate specificity, cleavage pattern, thermoactivation, thermostability, pH dependent activity or optimum, pH dependent stability, temperature dependent activity or optimum, transglycosylation activity, stability, and any other parameter of interest, may be performed on purified variants in accordance with methods known in the art as described below.

#### 20 Degradation of $\beta$ -limit dextrin by maltogenic amylase variants.

Another important parameter in the evaluation of the substrate specificity of maltogenic amylase variants may be the degree to which such enzymes are capable of degrading starch that has been exhaustively treated with the exoglycosylase  $\beta$ -amylase. To screen for variants which show patterns of degradation on such a substrate differing from the patterns produced by the parent maltogenic amylase the following assay is performed:  $\beta$ -limit dextrin is prepared by incubating 25 ml 1% amylopectin in McIlvane buffer (48.5 mM citrate and 193 mM sodium phosphate pH 5.0) with 24  $\mu$ g/ml  $\beta$ -amylase overnight at 30°C. Unhydrolysed amylopectin (i.e.,  $\beta$ -limit dextrin) is precipitated with 1 volume 95% ethanol, washed and redissolved in water. 1 ml  $\beta$ -limit dextrin is incubated with 18  $\mu$ l enzymes (at 2.2 mg/ml) and 100  $\mu$ l 0.2 M citrate-phosphate pH 5.0 for 2 hrs at 30°C and analysed by HPLC as described above. Total hydrolysis of  $\beta$ -limit dextrin is

carried out in 2M HCl at 95°C. The concentration of reducing ends is measured by methods known in the art.

#### Calcium binding affinity

Unfolding of maltogenic amylases by exposure to heat or to denaturants such as guanidine hydrochloride is accompanied by a decrease in fluorescence, and loss of calcium ions leads to unfolding. Thus, the affinity of a maltogenic amylase variant for calcium can be measured by fluorescence measurements before and after incubation of the variant (e.g., at a concentration of 10 mg/ml) in a buffer (e.g., 50 mM HEPES, pH 7) with different concentrations of calcium (e.g., in the range from 1 mM-100 mM) or of EGTA (e.g., in the range from 1-1000 mM) for a sufficiently long period of time (such as 22 hours at 55°C).

The measured fluorescence,  $F$ , is composed of contributions from the folded and unfolded forms of the enzyme. The following equation can be derived to describe the dependence of  $F$  on calcium concentration ( $[Ca]$ ):

$$F = [Ca]/(K_{diss} + [Ca])(a_u - b_u \log([Ca])) + K_{diss}/(K_{diss} + [Ca])(a_0 - b_0 \log([Ca]))$$

where  $a_u$  is the fluorescence of the native (folded) form of the enzyme,  $b_u$  is the linear dependence of  $a_u$  on the logarithm of the calcium concentration (as observed experimentally),  $a_0$  is the fluorescence of the unfolded form and  $b_0$  is the linear dependence of  $a_0$  on the logarithm of the calcium concentration.  $K_{diss}$  is the apparent calcium binding constant for an equilibrium process as follows:



In fact, unfolding proceeds extremely slowly and is irreversible. The rate of unfolding is dependent on calcium concentration, and such dependency for a given enzyme provides a measure of the calcium binding affinity of the enzyme. By defining a standard set of reaction conditions (e.g., 22 hours at 55°C), a meaningful

comparison of  $K_{0.5}$  for different maltogenic amylase variants can be made.

#### Determination of maltogenic amylase in MANU

One Maltogenic Amylase Novo Unit (MANU) is the amount of enzyme which under standard will cleave one  $\mu\text{mol}$  maltotriose per minute. The standard conditions are 10 mg/ml maltotriose, 37°C, pH 5.0, 30 minutes reaction time.

The pH dependence is found by repeating this measurement at the same conditions, but at different pH values.

#### EXAMPLES

##### EXAMPLE 1

Construction of a variant of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 with altered pH dependent activity

The maltogenic amylase having the amino acid sequence shown as amino acids 1-686 is expressed in *Bacillus subtilis* from a plasmid denoted herein as pLBei010. This plasmid contains *amyM* in which the expression of *amyM* is directed by its own promoter and the complete gene encoding the maltogenic amylase, e.g., as contained in the strain DSM 11837. The plasmid contains the origin of replication, *ori*, from plasmid pUB110 and an kanamycin resistance marker for selection purposes.

##### 25 Primer sequences

Site directed mutants of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 were constructed by the megaprimer method essentially as described by Kammann et al. (1999). Briefly, a mutagenic oligonucleotide primer is used together in a PCR reaction with a suitable opposite DNA strand end primer to create a preliminary PCR product. This product is then used as a megaprimer together with another opposite DNA strand end primer to create a double-stranded DNA product. The product of the final PCR reaction was

routinely used to replace a corresponding DNA fragment in the pLBei010 plasmid by standard cloning procedures. Mutants were transformed directly into *Bacillus subtilis* strain SHa273, a derivative of *Bacillus subtilis* 168 which is apr<sup>-</sup>, npr<sup>-</sup>, amyE<sup>-</sup>, amyR2<sup>-</sup> and prepared by methods known in the art.

Oligonucleotide primers used in the construction of described variants are as listed below:

Variant Sequence (5' → 3')

10 F188H: SEQ ID NO: 3  
F188E: SEQ ID NO: 4  
F284E: SEQ ID NO: 5  
F284D: SEQ ID NO: 6  
F284K: SEQ ID NO: 7  
15 N327D: SEQ ID NO: 8

Variant Sequence (3' → 5')

T288K: SEQ ID NO: 9  
T288R: SEQ ID NO: 10

20 Aspartate variants of F284, T288 and N327 were obtained using primer A189 (SEQ ID NO: 11) and B649 (SEQ ID NO: 12) as end-primers.

F188-variants F188L, T189Y were obtained using primer A82 (SEQ ID  
25 NO: 13) and B346 (SEQ ID NO: 14) as end-primers.

PCR products with the desired modification(s) were purified, digested with appropriate enzymes, separated by agarose gel electrophoresis and extracted, ethanol precipitated in the  
30 presence of glycogen, resuspended in H<sub>2</sub>O, ligated to pLBei010 which had been digested with the same appropriate enzymes, and transformed into *Bacillus subtilis* SHa273. Transformants were checked for size by colony PCR and for the insertion or removal of specific restriction sites by restriction enzyme digestion.  
35 Positive colonies were verified by DNA sequencing methods as described in the art.

### Fermentation

The *B. subtilis* SHa273 mutant clones were grown overnight on LB-Kana (10 µg/ml)-Starch plates at 37°C. The colonies from the plate were resuspended in 10 ml Luria broth. One-sixth of each of the suspensions were inoculated into a 500 ml shake flasks containing 100 ml PS-1 media, a soy meal/sucrose-based media, kanamycin for a final concentration of 10 µg/ml and 100 µl 5M NaOH. The pH was adjusted to 7.5 with NaOH before inoculation. The cultures were incubated for five days at 30°C with shaking at 270-300 rpm.

### Enzyme Purification

Large particles from the media were removed by flocculation before affinity chromatography. Superfloc C521 (American Cyanamide Company) was used as the cationic flocculant and Superfloc A130 (American Cyanamide Company) as the anionic flocculant.

The culture suspension was diluted 1:1 with deionized water and the pH was adjusted to approx. 7.5. A volume of 0.01 ml of 50% CaCl<sub>2</sub> per ml diluted culture was added during stirring. A volume of 0.015 ml of 20% Na-aluminate per ml diluted culture was titrated with 20% formic acid, while keeping the pH between 7 and 8. While stirring 0.025 ml 10% of C521 per ml diluted culture was added, followed by 0.05 ml 1% A130 per ml diluted culture, or until flocculation was observed. The solution was centrifuged at 4500 rpm for 30 minutes. Filtration was performed using a filter of pore size of 0.45 µm to exclude larger particles and any remaining bacteria. The filtered solution was stored at -20°C.

### Immobilization of $\alpha$ -cyclodextrin to DSV-agarose

One hundred mg of  $\alpha$ -cyclodextrin of molecular weight 972.86g/mol (Fluka 28705) was dissolved in 20 ml coupling buffer (0.5M Na<sub>2</sub>CO<sub>3</sub>, pH 11). Ten ml of DSV-agarose (Mini-Leak, Medium 10-20 mmol/l of divinyl sulfone activated agarose (Kem-En-Tec) was washed thoroughly with deionized water, then dried by suction and transferred to the  $\alpha$ -cyclodextrin solution. After the mixture had

stirred for 24 hr at ambient temperature, the gel was washed with deionized water, followed by 0.5M  $\text{KHCO}_3$ . The gel was transferred to the blocking buffer (20ml 0.5M  $\text{KHCO}_3$  + 1ml mercaptoethanol), stirred for 2 hr at ambient temperature, then washed with  
5 deionized water.

#### Affinity chromatography

The variants were purified by affinity chromatography using the Pharmacia FPLC System. A 0.04 volume of 1M Na-acetate pH 5 was added to the filtrate obtained by flocculation to adjust pH and  
10  $\text{CaCl}_2$  was added to a final concentration of  $10^{-10}$  M. The solution was filtered and degassed. A Pharmacia XK16 column was prepared with ten ml of the immobilised  $\alpha$ -cyclodextrin, then equilibrated in the equilibration buffer (25 mM Na-acetate pH 5) by washing with approximately 10 times the column volume. The filtrate was  
15 applied to the XK16 column, which was then washed with the equilibration buffer until protein could no longer be detected in the washing buffer. The column was washed with the equilibration buffer containing 0.5M NaCl to elute nonspecific material, followed by another wash with 2-3 times the column volume of the  
20 equilibration buffer. All washings were performed using a flow rate of 10ml/min. Specifically bound material was eluted using a solution of 2%  $\alpha$ -cyclodextrin in the wash buffer and collected using the Pharmacia Liquid Chromatography Collector LCC-500 Plus using a flow rate of 5 ml/min.

25

#### **EXAMPLE 2**

##### **pH dependent activity of variants**

The variants prepared in the preceding Example were tested for activity at various pH values as follows.

30

A colorimetric glucose oxidase-peroxidase assay for liberated glucose from maltotriose or amylopectin was used to determine the pH activity profiles of the enzyme variants (Glucose/GOD-Perid<sup>®</sup> Method, Boehringer Mannheim, Indianapolis IN). Activity was

assayed in a buffer of 25 mM citrate-phosphate, 0.1mM CaCl<sub>2</sub> at pH values of 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.6. The buffer pH was adjusted using NaOH and enzymes were diluted in 25 mM citrate-phosphate buffer pH 5. Measurements were taken in duplicate to obtain an average value. All values are relative to the pH at which the highest level of activity is seen.

The results, shown in the table below, indicate that each of the variants has an alteration in the pH dependent activity profile when compared to the parent maltogenic amylase. The highest level of activity for each variant is designated 100% and the activity of that variant measured at the other indicated pH values is a relative percentage of that maximum.

Modifications	pH															
	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.6		
None (parent)	0	0	0	8	47	80	100	95	91	80	66	39	35	30		
F188H	1	0	0	1	3	29	77	99	100	88	59	39	31	27		
F188E	0	0	0	2	27	62	89	100	93	71	46	28	20	18		
T268R	0	0	0	8	51	77	94	100	86	73	50	34	27	12		
N327D	1	1	7	27	67	95	100	98	77	33	19	11	5	0		

Further, a number of maltogenic amylase variants were tested for activity at pH 4.0 and 5.0, taking the activity of the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 at the same pH as 100 %. The activity was determined by hydrolysis of maltotriose (10 mg/ml) at 60°C, 50 mM

sodium acetate, 1 mM  $\text{CaCl}_2$ . The results are expressed as the ratio between activity at pH 5.0 and pH 4.0:

Modifications	pH 5.0/pH 4.0
N131D	0.24
I174Q	0.31
G397P	0.40
H103Y	0.40
$\Delta$ 262-266	0.47
T142A + D261G + T288P + Q449R	0.50
S32Q	0.53
S32D	0.55
T142A+ D261G	0.62
G370N+ N371G	0.66
S32N	0.68
N176S	0.79
D17E	0.80
None (parent)	1
$\Delta$ 191	1.39
192-A-193	1.61
I174E	1.80
192-A-G-193	1.90
$\Delta$ 192	2.22
F188L + D261G + T288P	2.47

s The results demonstrate that variants with a higher or lower pH optimum can indeed be obtained.

## EXAMPLE 3

## Thermostability of variants

Incubation at 80°C

The thermostability of a number of maltogenic amylase variants was tested by incubating an aqueous solution at 80°C, pH 4.3, 50 mM acetate buffer, 1mM CaCl<sub>2</sub>, and measuring the residual amylase activity at various times. The parent enzyme, the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1, was included for comparison. The results are expressed as residual activity at various times in percent of initial activity:

Variant	0	5 min.	10 min.	15 min.	20 min.	25 min.
None (parent)	100	23	9	3	1	0
A197P + D261G + T288P + N342S	100	36	28	14	16	9
A30D+ K40R+ D261G	100	38	24	15	13	10
T288K	100	64	31	18	7	4
T142A+ N327S+ K425E+ K520R+ N595I	100	47	39	25	19	11
T142A + D261G + T288P + Q449R	100	45	36	27	16	9
K40R+ F188L+ D261G+ A483T	100	56	48	40	36	30
F188L+ V336L+ T525A	100	63	49	48	52	47
F188I+ Y422F+ I660V	100	71	60	51	43	38
N115D+ F188L	100	73	60	51	44	39
F188L+ D261G+ T288P	100	60	67	66	63	67
F188L + D261G + T288P + A483T	100	66	72	73	75	78

N26S + F188L + D261G + T288P + T594A + I600V	100	80	80	82	84	84
N26S + T80A + F188L + D261G + T288P + R291L	100	80	75	82	83	87

The above data show a clearly improved thermostability for the variants compared to the parent amylase. Thus, after 15 minutes incubation at 80°C, a number of variants show at least 25 % residual activity, and some even show at least 50 % residual activity, whereas the parent enzyme has essentially lost its activity.

#### Incubation at 85°C

The variant S32E was tested by incubation with 1 mM Ca<sup>++</sup> at 85°C for 15 minutes. The variant showed a residual activity of 48 % whereas the parent enzyme (the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1) showed 32 % residual activity at the same conditions.

#### Incubation at 90°C

Four variants and the parent enzyme were tested by incubating at 90°C, pH 5.0, 50 mM acetate buffer, 1 mM CaCl<sub>2</sub>, and measuring the residual activity. The results were as follows:

Variant	0	10 min.	20 min.	30 min.
None (parent)	100	5	0	0
F188L + D261G + T288P	100	70	41	28
N26S + F188L + D261G + T288P + T594A + I600V	100	71	54	39
N26S + T80A + F188L + D261G + T288P + R291L	100	43	26	13
F188L + D261G + T288P + A483T	100	54	39	26

The variants show a clearly improved thermostability. Thus, the variants retain more than 10 % (or even more than 20 %) relative activity after 30 minutes incubation at 90°C, whereas the parent enzyme loses all activity after 20 minutes.

#### 5 DSC

Further, the thermostability was tested for some variants by DSC (differential scanning calorimetry) at pH values in the range 4.0-5.5. Again, the parent enzyme (the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1) was included for comparison. The results are expressed as the denaturation temperature (T<sub>m</sub>) at the given pH:

Modifications	pH 4.0	pH 4.3	pH 5.0	pH 5.5
None (parent)	64°C	79°C	83°C	88°C
N115D+ F188L		86°C		92°C
T142A+ N327S+ K425E+ K520R+ N595I				93°C
F188L + D261G + T288P	75°C		95°C	

The results show improved thermostability for each variant. One variant shows an improvement of more than 10°C at pH 4.0 and 5.5.

#### EXAMPLE 4

##### Specific activity of variants

Amylase activity was determined by a colorimetric measurement after action on Phadebas tablets at pH 5.0 and 60°C. The results for two variante, relative to the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 were as follows:

Modificatio ns	Relative amylase activity
-------------------	------------------------------

None (parent)	100
192-A-193	110
Δ (191-195)	300

The specific activity was further tested by action on maltotriose at pH 4.0, 60°C by the MANU method described above. The results showed that the variant G370N, N371G has a maltotriose activity of 106 % compared to the parent maltogenic amylase.

#### EXAMPLE 5

##### Inhibition of retrogradation

The efficiency of the parent maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 and variants thereof to inhibit retrogradation was determined as follows:

730 mg of 50 % (w/w) amylopectin slurry in 0.1 M sodium acetate, at a selected pH (3.7, 4.3 or 5.5) was mixed with 20 µl of an enzyme sample, and the mixture was incubated in a sealed ampoule for 1 hour at 40°C, followed by incubation at 100 °C for 1 hour in order to gelatinize the samples. The sample was then aged for 7 days at room temperature to allow recrystallization of the amylopectin. A control without enzyme was included.

After aging, DSC was performed on the sample by scanning from 5°C to 95°C at a constant scan rate of 90°C/hour. The area under the first endothermic peak in the thermogram was taken to represent the amount of retrograded amylopectin, and the relative inhibition of retrogradation was taken as the area reduction (in %) relative to the control without enzyme.

In the table below, the efficiency of the enzyme is expressed as the ratio of the relative inhibition of retrogradation to the enzyme dosage (in MANU/ml):

pH	Modifications	MANU/ml	Relative inhibition	Efficiency
3.7	A30D+ K40R+ D261G	0.23	0.38	1.7
3.7	T142A+ N327S+ K425E+ K520R+ N595I	0.07	0.29	4.1
3.7	None (parent)	0.27	0.38	1.4
4.3	N115D+ F188L	0.01	0.18	18
4.3	None (parent)	0.27	0.43	1.6
5.5	$\Delta$ (191-195)+ F188L+ T189Y	0.02	0.12	6
5.5	$\Delta$ (191-195)	0.02	0.14	7
5.5	$\Delta$ (191-195)	0.05	0.31	6.2
5.5	N115D+ F188L	0.01	0.39	39
5.5	T142A+ D261G	0.14	0.53	3.8
5.5	None (parent)	0.27	0.49	1.8

The results demonstrate that a number of variants are more efficient than the parent amylase to inhibit retrogradation.

#### Example 6

##### 5 Substrate specificity of variants

The activity of variants was tested on two different substrates: glucose release from maltotriose and color release from Phadebas colored starch. The parent enzyme (the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1) was tested for comparison. The measurements were made at pH 5, and each activity was expressed relative to the parent enzyme. The ratio of activities on the two substrates was found to be as follows:

Variant	Activity ratio Starch/maltotriose
Parent enzyme	1.0

F188L, D261G, T288P	3.6
F188L, D261G, T288P, T594A, I600V	5.5
N26S, T80A, F188L, D261G, T288P, R291L	1.9
A197P, D261G, T288P, N342S	1.5
T142A, D261G, T288P, Q449R	2.5
F188L, D261G, T288P, A483T	2.5

It can be seen that the 6 variants have an increased activity on starch relative to maltotriose.

#### 5 EXAMPLE 7

##### Preparation of an enzymatically modified starch

10 MANU/g DS variant (the maltogenic amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 with F188L+D261G+T288P substitutions) is added to a 30% (by weight of starch in water) suspension. The suspension is heated with direct  
 10 steam (3°C per minute) to 85°C, where after the starch solution obtained is maintained at 85°C for 10 minutes. The converted starch solution obtained then passed through a jet-cooker where the enzyme is inactivated at 140°C for 45 seconds. The starch  
 15 solution obtained is then diluted with water to about 20% by weight of dry substance and cooled to 50°C. The viscosity is then measured (Brookfield, Type LVT, 30 rpm) directly upon the preparation of the solution and subsequently after 1,2,4, and 24 hours storage in an oven.

20

#### EXAMPLE 8

##### Surface-sizing of Paper

An enzymatically modified starch solution prepared in accordance with Example 9 is used as surface-sizing agent for paper. The  
 25 modified starch solution is applied to a base paper in the form of an aqueous solution by means of a horizontal size press (type

T.H. Dixon; model 160-B; roll hardness 80 shore). The surface-sized paper is then dried with an air foil drier to a moisture content of 5% by weight.

5 **EXAMPLE 9**

**High Purity Maltose production using a maltogenase variant**

1400 g of waxy maize starch (American Maize Co.) (90% DS = 1250 g) is suspended in 3600 g de-ionized water to make a 25% DS slurry. pH is adjusted to 5.1. 8 g of a maltogenic amylase  
10 variant having an activity of 1500 MANU/g is added to the slurry. The definition of the enzyme activity unit MANU appears from B547F-GB, which is obtainable on request from Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark. As a substrate in relation to the activity determination, carried out at pH 5 and  
15 at 37°C, is used maltotriose, which is degraded to glucose or maltose, which again is determined spectrophotometrically.

The slurry is incubated in a water bath at 70°C for 46 hours. The reactor is coupled to an ultrafiltration module (pump:  
20 Eagholm, type BF 471M44; module: Mini-Lab 10, DDS RO-Divison, Denmark; UF membranes: Dow Denmark, Type GR50PP, cut off 2000; filtration area: 0.0336 m<sup>2</sup>), and during the incubation, the reaction mixture is pumped through the membrane module with a flow of 440 l/hour giving rise to a retentate and permeate.  
25 During the reaction time 3000 ml of permeate is collected giving a permeate flux of 2000 ml/hour/m<sup>2</sup>. The initial inlet pressure is 2.1 bar and due to increase of concentration and viscosity of the reaction 2200 ml of deionized water is added from time to time in order to keep the DS content in the reaction mixture at  
30 a reasonable level (about 25% DS).

The DS content and the density in the permeate is determined. The profile of the product is determined by HPLC.

35 40 g of the above-indicated retentate is centrifuged at 4,000 rpm for 40 minutes. The precipitate and the supernatant from the centrifugation were separated and the precipitate was washed 4 times with de-ionized water. The washed precipitate is dried in

an oven at 50°C over night. The precipitate is characterized by NMR (Nuclear Magnetic Resonance) spectroscopy and GPC (Gel Permeation Chromatography). For the NMR experiment 15 mg of the precipitate is dissolved in 0.5 ml DMSO-d<sub>6</sub> (DMSO is an abbreviation for Dimethyl Sulfoxide, and d<sub>6</sub> signifies that 6 deuterium atoms are present in the DMSO molecule instead of 6 hydrogen atoms) by heating to about 50°C for about 1/2 hour. The NMR experiments are performed at 60°C using a Bruker AC 300 spectrometer. The  $\alpha$ -1,4/ $\alpha$ -1,6 ratio is determined by integration of signals from the  $\alpha$ -1,4 linkages (5.2 ppm), the  $\alpha$ -ends (5.1 ppm), the  $\alpha$ -1,6 linkages (4.65 ppm) and the  $\beta$ -ends (4.35 ppm).

For the GPC experiment 10 mg of the precipitate is dissolved in 4 ml DMSO. The samples were analyzed on three PLGel 20  $\mu$ m MIXED A (300 x 7.5 mm) columns (from Polymer Laboratories, England) in series using Waters HPLC (High Performance Liquid Chromatography) equipment.

The molecular weight distribution of the samples is calculated from a calibration curve based on pullulan standards.

#### EXAMPLE 10

1200 g of common corn starch (with a % DS of 90, i.e., corresponding to 1000 g of dry starch) is suspended in 4000 g deionized water to make a 20 % DS slurry. pH is adjusted to 5.1. 7 g of a maltogenic amylase variant having an activity of 1500 MANU/g is added to the slurry.

The slurry is incubated in a water bath at 70°C for 46 hours. The reactor is coupled to an ultrafiltration module (pump: Bagholm, type BF 471M44; module: Mini-Lab 10, DDS RO-Division, Denmark; UF membranes: Dow Denmark, Type GR90PP, cut off 2000; filtration area: 0.0336 m<sup>2</sup>), and during the incubation, the reaction mixture is pumped through the membrane module with a flow of 540 l/hour giving rise to a retentate and a permeate. During the reaction time 1845 ml of permeate is collected giving a permeate flux of 1194 ml/hour/m<sup>2</sup>. The initial inlet pressure is 1.8 bar, and due to increase of concentration and viscosity

of the reaction mixture, the inlet pressure at the end of the reaction is 3.4. bar. During the reaction about 1000 ml of deionized water is added from time to time in order to keep the DS content in the reaction mixture at about 20% DS. At the end  
5 of the reaction the DS content in the reaction mixture is determined.

The DS content and the density in the permeate are determined by HPLC.

10

40.0 g of the above-indicated retentate is centrifuged at 4,000 rpm for 40 minutes. The precipitate and the supernatant are separated and the precipitate is washed 4 times with deionized water. The washed precipitate is dried in an oven at 50°C over  
15 night. 0.9 g of precipitate is isolated.

The precipitate is characterized by NMR spectroscopy and GPC. For the NMR experiment 15 mg of the precipitate is dissolved in 0.5 ml DMSO-d<sub>6</sub> by heating to about 50°C for about 1/2 hour. The  
20 NMR experiments are performed at 60°C using a Bruker AC 300 spectrometer. The  $\alpha$ -1,4/ $\alpha$ -1,6 ratio is determined by integration of signals from the  $\alpha$ -1,4 linkages (5.2 ppm), the  $\alpha$ -ends (5.1 ppm), the  $\alpha$ -1,6 linkages (4.65 ppm) and the  $\beta$ -ends (4.35 ppm).

25 For the GPC experiment 10 mg of the precipitate is dissolved in 4 ml DMSO. The samples are analyzed on three PLGel 20  $\mu$ m MIXED A (300 x 7.5 mm) columns in series using Waters HPLC equipment.

The molecular weight distribution of the samples is calculated  
30 from a calibration curve based on pullulan standards.

#### EXAMPLE 11

A 35 % dry solid starch (common corn starch) slurry was liquefied using a pilot plant jet cooking equipment (Hydroheater  
35 model). The flow was adjusted to 500 ml/min corresponding to a residence time of 5 minutes in the jets holding tubes. Jetting conditions : pH 6.0, Enzyme dosage 60 NU (Termamyl LC)/ g DS,

Ca-level 5 ppm. Temperature 105 °C.

When the flow rate and temperature have been constant for about 15 minutes, 2 liters of starch slurry is collected after the jets flash cooler. The starch slurry is transferred to laboratory flasks; pH is adjusted to 2.5 and kept for 10 minutes in an oil bath in order to completely inactivate the enzymes.

The dextrose equivalent (DE) is measured.

10

pH in the slurry is adjusted to 5.0, the temperature lowered to 90 °C and a maltogenic amylase variant MV 447 added (dose 10 MANU/g DS). Samples are taken after 1,2,4,6 and 24 hours and the sugar profile determined by HPLC.

15

#### EXAMPLE 12

A 35 % dry solid starch (common corn starch) slurry was liquefied using a pilot plant jet cooking equipment (Hydroheater model). The flow was adjusted to 500 ml/min corresponding to a residence time of 5 minutes in the jets holding tubes. Jetting conditions : pH as supplied, Temperature 150 °C.

When the flow rate and temperature have been constant for about 15 minutes, 2 liters of starch slurry is collected after the jets flash cooler.

25

The dextrose equivalent (DE) is measured.

pH in the slurry is adjusted to 5.0, the temperature lowered to 90 °C and a maltogenic amylase variant MV 447 added (dose 10 MANU/g DS). Samples are taken after 1,2,4,6 and 24 hours and the sugar profile determined by HPLC.

30

#### EXAMPLE 13

A 35 % dry solid starch (common corn starch) slurry was liquefied using a pilot plant jet cooking equipment (Hydroheater model). The flow was adjusted to 500 ml/min corresponding to a residence time of 5 minutes in the jets holding tubes. Jetting

35

conditions : pH 6.0, Enzyme dosage 60 NU (Termamyl LC)/ g DS, Ca-level 5 ppm, Temperature 105 °C.

When the flow rate and temperature have been constant for about 15 minutes, 2 liters of starch slurry is collected after the  
5 jets flash cooler. The starch slurry is transferred to a laboratory flask (reactor); pH is adjusted to 2.5 and kept for 10 minutes in an oil bath in order to completely inactivate the enzymes.

10 The dextrose equivalent (DE) is measured.

pH in the slurry is adjusted to 5.0, the temperature lowered to 90 °C and a maltogenic amylase variant MV 447 added (dose 10 MANU/g DS).

15

After 2 hours of incubation the starch slurry is diluted to 25% DS. The temperature is lowered to 70 °C. The reactor is coupled to an ultrafiltration module (pump: Eagholm, type BF 471M44; module: Mini-Lab 10, DDS RO-Divison, Denmark; UF membranes: Dow  
20 Denmark, Type GR90PP, cut off 2000; filtration area: 0.0336 m<sup>2</sup>), and during the incubation, the reaction mixture is pumped through the membrane module with a flow of 440 l/hour giving rise to a retentate and permeate. During the reaction time the permeate is collected. The initial inlet pressure is 2.1 bar and  
25 due to increase of concentration and viscosity of the reaction de-ionized water is added from time to time in order to keep the DS content in the reaction mixture at a reasonable level (about 25% DS).

30 The DS content and the density in the permeate is determined. The profile of the product is determined by HPLC.

40 g of the above-indicated retentate is centrifuged at 4,000 rpm for 40 minutes. The precipitate and the supernatant from the  
35 centrifugation were separated and the precipitate was washed 4 times with de-ionized water. The washed precipitate is dried in an oven at 50°C over night. The precipitate is characterized by NMR (Nuclear Magnetic Resonance) spectroscopy and GPC (Gel

Permeation Chromatography). For the NMR experiment 15 mg of the precipitate is dissolved in 0.5 ml DMSO- $d_6$  (DMSO is an abbreviation for Dimethyl Sulfoxide, and  $d_6$  signifies that 6 deuterium atoms are present in the DMSO molecule instead of 6 hydrogen atoms) by heating to about 50°C for about 1/2 hour. The NMR experiments are performed at 60°C using a Bruker AC 300 spectrometer. The a-1,4/a-1,6 ratio is determined by integration of signals from the a-1,4 linkages (5.2 ppm), the a-ends (5.1 ppm), the a-1,6 linkages (4.65 ppm) and the b-ends (4.35 ppm).

10

For the GPC experiment 10 mg of the precipitate is dissolved in 4 ml DMSO. The samples were analyzed on three PLGel 20  $\mu$ m MIXED A (300 x 7.5 mm) columns (from Polymer Laboratories, England) in series using Waters HPLC (High Performance Liquid Chromatography) equipment.

15

The molecular weight distribution of the samples is calculated from a calibration curve based on pullulan standards.

#### EXAMPLE 14

20 A 35 % dry solid starch (common corn starch) slurry was liquefied using a pilot plant jet cooking equipment (Hydroheater model). The flow was adjusted to 500 ml/min corresponding to a residence time of 5 minutes in the jets holding tubes. Jetting conditions : pH as supplied, Temperature 150 °C.

25

When the flow rate and temperature have been constant for about 15 minutes, 2 liters of starch slurry is collected after the jets flash cooler.

30 The dextrose equivalent (DE) is measured.

pH in the slurry is adjusted to 5.0, the temperature lowered to 90 °C and a maltogenic amylase variant MV 447 added (dose 10 MANU/g DS).

35

After 2 hours of incubation the starch slurry is diluted to 25% DS. The temperature is lowered to 70 °C. The reactor is coupled

to an ultrafiltration module (pump: Eagholt, type BF 471M44; module: Mini-Lab 10, DDS RO-Divison, Denmark; UF membranes: Dow Denmark, Type GR90PP, cut off 2000; filtration area: 0.0336 m<sup>2</sup>), and during the incubation, the reaction mixture is pumped  
5 through the membrane module with a flow of 440 l/hour giving rise to a retentate and permeate. During the reaction time the permeate is collected. The initial inlet pressure is 2.1 bar and due to increase of concentration and viscosity of the reaction de-ionized water is added from time to time in order to keep the  
10 DS content in the reaction mixture at a reasonable level (about 25% DS).

The DS content and the density in the permeate is determined. The profile of the product is determined by HPLC.

15 40 g of the above-indicated retentate is centrifuged at 4,000 rpm for 40 minutes. The precipitate and the supernatant from the centrifugation were separated and the precipitate was washed 4 times with de-ionized water. The washed precipitate is dried in  
20 an oven at 50°C over night. The precipitate is characterized by NMR (Nuclear Magnetic Resonance) spectroscopy and GPC (Gel Permeation Chromatography). For the NMR experiment 15 mg of the precipitate is dissolved in 0.5 ml DMSO-d<sub>6</sub> (DMSO is an abbreviation for Dimethyl Sulfoxide, and d<sub>6</sub> signifies that 6  
25 deuterium atoms are present in the DMSO molecule instead of 6 hydrogen atoms) by heating to about 50°C for about 1/2 hour. The NMR experiments are performed at 60°C using a Bruker AC 300 spectrometer. The a-1,4/a-1,6 ratio is determined by integration of signals from the a-1,4 linkages (5.2 ppm), the a-ends (5.1  
30 ppm), the a-1,6 linkages (4.65 ppm) and the b-ends (4.35 ppm).

For the GPC experiment 10 mg of the precipitate is dissolved in 4 ml DMSO. The samples were analyzed on three PLGel 20 µm MIXED A (300 x 7.5 mm) columns (from Polymer Laboratories, England) in  
35 series using Waters HPLC (High Performance Liquid Chromatography) equipment.

The molecular weight distribution of the samples is calculated

from a calibration curve based on pullulan standards.

#### EXAMPLE 15

A 35 % dry solid starch (common corn starch) slurry was  
5 liquefied using a pilot plant jet cooking equipment (Hydroheater  
model). The flow was adjusted to 500 ml/min corresponding to a  
residence time of 5 minutes in the jets holding tubes. Jetting  
conditions : pH 6.0, Enzyme dosage 60 NU (Termamyl LC)/ g DS,  
Ca-level 5 ppm, Temperature 105 °C.

10

When the flow rate and temperature have been constant for about  
15 minutes, 2 liters of starch slurry is collected after the  
jets flash cooler. The starch slurry is transferred to  
laboratory flasks; pH is adjusted to 2.5 and kept for 10 minutes  
15 in an oil bath in order to completely inactivate the enzymes.

The dextrose equivalent (DE) is measured.

pH in the slurry is adjusted to 5.0, the temperature lowered to  
20 90 °C and a maltogenic amylase variant MV 447 added (dose 10  
MANU/g DS). After 2 hours reaction time the temperature is  
lowered to 60 °C and a debranching enzyme added (Promozyme D,  
dose: 1NPUN/g DS). Samples are taken after 0,1,2,4,6,24 hours  
and the sugar profile determined by HPLC.

25

#### EXAMPLE 16

A 35 % dry solid starch (common corn starch) slurry was  
liquefied using a pilot plant jet cooking equipment (Hydroheater  
model). The flow was adjusted to 500 ml/min corresponding to a  
30 residence time of 5 minutes in the jets holding tubes. Jetting  
conditions : pH as supplied, Temperature 150 °C.

When the flow rate and temperature have been constant for about  
15 minutes, 2 liters of starch slurry is collected after the  
35 jets flash cooler.

The dextrose equivalent (DE) is measured.

pH in the slurry is adjusted to 5.0, the temperature lowered to 90 °C and a maltogenic amylase variant MV 447 added (dose 10 MANU/g DS). After 2 hours reaction time the temperature is lowered to 60 °C and a debranching enzyme added (Promozyme D, dose: 1NPUN/g DS). Samples are taken after 0,1,2,4,6,24 hours and the sugar profile determined by HPLC.

## CLAIMS

1. A method for preparing maltose and/or a modified starch  
5 comprising the following steps:
- 1) treating starch with a variant of a maltogenic amylase  
having the amino acid sequence shown in position 1-686 of  
SEQ ID NO: 1, where the variant
- 10 a) has maltogenic amylase activity;  
b) has at least 70% identity to position 1-686 of SEQ  
ID NO: 1, and  
c) has optimum maltogenic amylase activity in the pH  
15 range 3.5-7.0; and
- II) optionally recovering the maltose and/or the modified  
starch.
- 20 2. The method according to claim 1, wherein the treated starch  
is raw starch.
3. The method according to claim 2, wherein the raw starch is  
waxy maize starch.
- 25 4. The method according to claims 2 or 3, wherein the treatment  
with the maltogenic amylase variant is carried out at a  
temperature, which is lower than the lowest temperature at which  
raw starch is gelatinized.
- 30 5. The method according to claim 4, wherein the treatment with  
the maltogenic amylase variant is carried out at a temperature  
in the range of from 55°C to 75°C.
- 35 6. The method according to any of claims 1-5, wherein the maltose  
and/or the modified starch are recovered.
7. The method according to claim 6, wherein the recovery of the

maltose and/or the modified starch is performed as an ultrafiltration, whereby the maltose is in the permeate, and the modified starch is produced as the solid phase by liquid-solid separation of the retentate.

5

8. The method according to claim 7, wherein the ultrafiltration is carried out simultaneously with the treatment of the raw starch with the maltogenic amylase variant, and that the temperature is above 40°C.

10

9. The method according to claim 8, wherein the temperature is above 50°C, preferably above 60°C, such as above 65°C, e.g. in the range of from 65°C to 75°C.

15 10. The method according to any of claims 1-9, wherein the modified starch is a limit dextrin.

11. Modified starch obtainable by the method defined in any of claims 1-10.

20

12. The method according to claim 1, wherein the treatment with the maltogenic amylase variant is carried out after gelatinisation of the starch.

25 13. The method according to claim 12, wherein the treatment with the maltogenic amylase variant is carried out at a temperature above 65°C, in particular above 70°C, such as above 75°C, e.g. above 80°C, preferably above 85°C, more preferably above 90°C, such as above 95°C.

30

14. The method according to claim 1, wherein the treatment with the maltogenic amylase variant is carried out during or after the liquefaction step.

35 15. The method according to any of claims any of claims 12-14, wherein said method comprises a saccharification step.

16. The method according to any of claims 1-15, for producing maltose syrup, such as a Low Maltose Syrup, a High Maltose Syrup, or an Ultra High Maltose Syrup, or Speciality Syrups.

17. The method according to any of claims 1-16, wherein the variant which shows a residual maltogenic amylase activity of at least 25%, such as at least 30%, e.g. at least 40%, preferably at least 50%, e.g. at least 60%, more preferably at least 70%, such as at least 80%, e.g. at least 90% after incubation with 1 mM Ca<sup>++</sup> at pH 4.3, at 80°C for 15 minutes.

18. The method according to any of claim 1-17, wherein the variant comprises a substitution of an amino acid residue corresponding to one or more of the following residues of the amino acid sequence set forth in SEQ ID NO: 1:  
L51, L71, S72, V74, L75, L78, T80, L81, G83, T84, D85, N86, T87, G88, Y89, H90, G91, T94, R95, D96, F97, V114, I125, V126, T134, G157, Y167, F168, H169, H170, N171, G172, D173, I174, S175, N176, D178, D179, R180, Y181, E182, A183, Q184, K186, N187, F188, T189, D190, A192, G193, F194, S195, L196, L217, S235, G236, V254, V279, V281, L286, V289, I290, V308, L321, I325, D326, L343, F349, S353, I359, I405, L448, Q449, L452, I470, G509, V515, S583, G625, L627, L628 or A670.

19. The method of any of claims 1-18, wherein the variant comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

L217 in combination with L75, L217F/Y in combination with L75F/Y, L51W, L71I, S72C, V74I, L75N/D/Q/I/V/F/Y, L75F/Y, L78N/I, L78I, T80I/L/V/S/N/G, L81I/V/S/T/N/Q/K/H, G83A/S/T/N/Q/E/D/R/H/L, T84S/A/N/D/G, D85A/T/S/N/G, N86Q/E/D/Y/H/K, T87S/I, G88A/V/T/S, Y89F, H90N/Q/K, G91A/T/S/V/N, T94N/D/A/M/V/I/L, T94V/I/L, R95K/Q, D96N/V/Q/I, F97Y, V114V/I/L, I125L/M/F/Y/W, V126I/L, T134V/I/L/M/F/Y/W, G157A/V/I/L, Y167F/R/C, F168Y, H169N/Q/K, H170N/Q/K, N171D/E/Q/H/R/K/G, G172A/T/S, D173N/S/T/Y/R/G, I174N/Q/L, S175T/A/N/D, N176S/T/H/Q/P, D178N/Q/E/K/H, D179Y/N/H, R180W,

Y181R/F/C/L, E182D, A183S/C/G, Q184E, K186R,  
N187Q/E/L/F/H/K/V/L, F188Y/L/I/H/N, T189N/D/A/S/H/Y/G,  
D190E/Q/H/N/K, A192T/D/E/N/K, G193A/S/T, F194Y, S195N/D/E/R/K/G,  
L196I, L217V/I/M/F/Y/W, S235I/L/M/F/Y/W, G236A/V/I/L/M/F/Y/W,  
5 V254I/L/M/F/Y/W, V279M/I/L/F, V281I/L/M/F/Y/W, L286F, V289I/L/R,  
I290M/L/F, V308I/L/M/F/Y/W, L321I/M/F/Y/W, I325L/M/F/Y/W,  
D326E/Q, L343M/F/Y/W, F349W/Y, S353V/I/L, I359L/M/F/Y/W,  
I405M/L/Y/F/W, L448Y, Q449Y, L452M/Y/F/W, I470M/L/F,  
G509A/V/I/L/M/S/T/D/N, V515I/L, S583V/I/L/V,  
10 G625A/V/I/L/M/F/Y/W, L627M/F/Y, L628M/I/F/Y/W or  
A670V/I/L/M/F/Y/W.

20. The method according to any of claims 1-19, wherein the  
maltogenic amylase variant shows an improved stability compared  
15 to the polypeptide of SEQ ID NO: 1.

21. The method according to any of claims 1-20, wherein the  
variant comprises one or more substitutions corresponding to the  
following substitutions in the amino acid sequence set forth in  
20 SEQ ID NO: 1:  
Q13, I16, D17, N26, N28, P29, A30, S32, Y33, G34, L35, K40, M45,  
P73, V74, D76, N77, D79, N86, R95, N99, I100, H103, Q119, N120,  
N131, S141, T142, A148, N152, A163, H169, N171, G172, I174,  
N176, N187, F188, A192, Q201, N203, H220, N234, G236, Q247,  
25 K249, D261, N266, L268, R272, N275, N276, V279, N280, V281,  
D285, N287, F297, Q299, N305, K316, N320, L321, N327, A341,  
N342, A348, Q365, N371, N375, M378, G397, A381, F389, N401,  
A403, K425, N436, S442, N454, N468, N474, S479, A483, A486,  
V487, S493, T494, S495, A496, S497, A498, Q500, N507, I510,  
30 N513, K520, Q526, A555, A564, S573, N575, Q581, S583, F586,  
K589, N595, G618, N621, Q624, A629, F636, K645, N664 or T681.

22. The method according to any of claims 1-21, wherein the  
variant comprises one or more substitutions corresponding to the  
35 following substitutions in the amino acid sequence set forth in  
SEQ ID NO: 1:  
K40, V74, H103, S141, T142, F188, H220, N234, K249, D261, L268,  
V279, N342, H344, G397, A403, K425, S442, S479, S493, T494,

S495, A496, S497, A498, Q500, K520, A555 or N595.

23. The method according to any of claims 1-22, wherein the variant comprises one or more substitutions corresponding to the following substitutions in the amino acid sequence set forth in SEQ ID NO: 1:

K40R, V74P, H103Y/V/I/L/F/Y, S141P, T142A, F188I/L, H220Y/L/M, N234P, K249P, D261G, L268P, V279P, N342P, H344E/Q/N/D/Y, G397P, A403P, K425E, S442P, S479P, S493P, T494P, S495P, A496P, S497P, A498P, Q500P, K520R, A555P or N595I.

24. The method according to any of claims 1-23, wherein the modification comprises an amino acid modification at a position corresponding to D17, N28, P29, A30, S32, Y33, G34, R95, H103, N131, H169, I174 and/or Q201 such as to improve calcium coordination, preferably a substitution corresponding to D17Q/E, A30D/M/L/A/V/I/E/Q, S32D/E/N/Q, R95M/L/A/V/I/E/Q, H103Y/N/Q/D/E, N131D, H169N/D/E/Q, I174E/Q, and or Q201E.

25. The method according to any of claims 1-24, wherein the modification comprises a substitution at a position corresponding to Q13, N26, N77, N86, N99, Q119, N120, N131, N152, N171, N176, N187, Q201, N203, N234, Q247, N266, N275, N276, N280, N287, Q299, N320, N327, N342, Q365, N371, N375, N401, N436, N454, N468, N474, Q500, N507, N513, Q526, N575, Q581, N621, Q624 and/or N664 such as to remove a deamidation site, preferably a substitution corresponding to Q13S/T/A/V/L/I/F/M, N26S/T/A/V/L/I, N77S/T/A/V/L/I, N86S/T/A/V/L/I, N99T/S/V/L, Q119T/S, N120S/T/A/V/L/I, N131S/T/A/V/L/I, N152T/S/V/L, N171Y/D/S/T, N176S/T/A/V/L/I, N187S/T/A/V/L/I, Q201S/T/A/V/L/I/F/M, N203D/S/T/A/V/L/I, N234S/T/A/V/L/I, Q247S/T/A/V/L/I/F/M, N266S/T/A/V/L/I, N275S/T/A/V/L/I, N276S/T/A/V/L/I, N280S/T/A/V/L/I, N287S/T/A/V/L/I, Q299L/T/S, N320S/T/A/V/L/I, N327S/T/A/V/L/I, N342S/T/A/V/L/I, Q365S/T/A/V/L/I, N371S/T/A/V/L/I, N375S/T/A/V/L/I, N401S/T/A/V/L/I, N436S/T/A/V/L/I, N454D/S/T/A/V/L/I, N468D/S/T/A/V/L/I, N474D/S/T/A/V/L/I, Q500S/T/A/V/L/I/F/M, N507S/T/A/V/L/I, N513S/T/A/V/L/I, Q526

D/S/T/A/V/L/I, N575S/T/A/V/L/I, Q581S/T/A/V/L/I/F/M,  
N621S/T/A/V/L/I Q624S/T/A/V/L/I/F/M and/or N664D/S/T/A/V/L/I.

26. The method according to any of claims 1-25, wherein the  
5 modification comprises a substitution at a position  
corresponding to I16, L35, M45, P73, D76, D79, A192, I100, A148,  
A163+G172, L268, V281, D285, L321, F297, N305, K316, S573, A341,  
M378, A381, F389, A483, A486, I510, A564, F586, K589, F636,  
K645, A629, and/or T681 such as to improve hydrogen bond  
10 contact, preferably a substitution corresponding to I16T/D/N,  
L35Q, M45K, P73Q, D76E, D79E/Y, A192S/D/N, I100T/S/D/N/E/Q,  
A148D/N/E/Q/S/T/R/K, A163Y+G172S/D/N, L268R/K, V281/Q, D285R/K,  
L321Q, F297N/D/Q/E, N305K/R, K316N/D, S573N/D, A341R/K, M378R/K,  
A381S/D/N, F389Y, A483S/D/N, A486Q/E, I510R/K, A564S/D/N,  
15 F586S/D/N, K589S/D/Q/N, F636Y, K645T, A629N/D/E/Q, and/or  
T681D/N/E/Q/S.

27. The method according to any of claims 1-26, wherein the  
modification comprises substitutions such as to introduce one or  
20 more interdomain disulfide bonds, preferably corresponding to  
G236C + S583C, G618C + R272C, and/or A348C + V487C.

28. The method according to any of claims 1-27, wherein the  
modification comprises a substitution at a position  
25 corresponding to L51, L75, L78, G88, G91, T94, V114, I125, V126,  
T134, G157, L217, S235, G236, V254, V279, V281, L286, V289,  
I290, V308, L321, I325, D326, L343, F349, S353, I359, I405,  
L448, Q449, L452, I470, G509, V515, S583, G625, L627, L628  
and/or A670 so as to fill an internal cavity or crevice,  
30 preferably a substitution corresponding to L51W, L75F/Y, L78I,  
G88A/V/T, G91T/S/V/N, T94V/I/L, V114V/I/L, I125L/M/F/Y/W,  
V126I/L, T134V/I/L/M/F/Y/W, G157A/V/I/L, L217V/I/M/F/Y/W,  
S235I/L/M/F/Y/W, G236A/V/I/L/M/F/Y/W, V254I/L/M/F/Y/W,  
V279M/I/L/F, V281I/L/M/F/Y/W, L286F, V289I/L/R, I290M/L/F,  
35 V308I/L/M/F/Y/W, L321I/M/F/Y/W, I325L/M/F/Y/W, D326E/Q,  
L343M/F/Y/W, F349W/Y, S353V/I/L, I359L/M/F/Y/W, I405M/L/Y/F/W,  
L448Y, Q449Y, L452M/Y/F/W, I470M/L/F, G509A/V/I/L/M/S/T/D/N,  
V515I/L, S583V/I/L/V, G625A/V/I/L/M/F/Y/W, L627M/F/Y,

L628M/I/F/Y/W, A670V/I/L/M/F/Y/W and/or L217 in combination with L75.

29. The method according to any of claims 1-28, wherein the  
5 modification comprises a substitution at a position corresponding to N106, N320 and/or Q624 so as to create a salt bridge, preferably a substitution corresponding to N106R, N320E/D and/or Q624E.

10 30. The method according to any of claims 1-29, wherein the modification comprises a substitution at a position corresponding to K244 and/or K316 such as to alter the charge distribution, preferably a substitution corresponding to K244S and/or K316G/N/D.

15 31. The method according to any of claims 1-30, wherein the modification comprises a substitution at a position corresponding to V281 and/or A629 such as to alter the binding site, preferably a substitution corresponding to V281Q and/or  
20 A629N/D/E/Q.

32. The method according to any of claims 1-31, wherein the modification comprises a substitution at a position corresponding to F143+F194+L78,  
25 A341+A348+L398+I415+T439+L464+L465, L557, S240+L268, Q208+L628, F427+Q500+N507+M508+S573 and/or I510+V620 such as to alter the interdomain interaction, preferably substitutions corresponding to F143Y+F194Y+L78Y/F/W/E/Q,  
A341S/D/N+A348V/I/L+L398E/Q/N/D+I415E/Q+T439D/E/Q/N+L464D/E+L465  
30 D/E/N/Q/R/K, L557Q/E/N/D, S240D/E/N/Q+L268D/E/N/Q/R/K, Q208D/E/Q+L628E/Q/N/D, F427E/Q/R/K/Y+Q500Y+N507Q/E/D+M508K/R/E/Q+S573D/E/N/Q, and/or I510D/E/N/Q/S+V620D/E/N/Q.

35 33. The method according to claim 1-32, wherein the variant comprises one or more of the following substitutions:  
N26S, T80A, T142A, F188L, A197P, D261G, T288P, R291L, N342S, Q449R, A483T, T594A, I600V (relative to SEQ ID NO: 1).

34. The method according to claim 33, wherein the variant comprises the following substitutions:

- F188L, D261G, and T288P,
- 5 -F188L, D261G, T288P, T594A and I600V,
- N26S, T80A, F188L, D261G, T288P and R291L,
- A197F, D261G, T288P, and N342S,
- T142A, D261G, T288P, and Q449R,
- F188L, D261G, T288P, and Q449R, or
- 10 -F188L, D261G, T288P, and A483T.

35. A modified starch obtainable by the method according to any of claims 12 to 34.

- 15 36. A process for surface-sizing and/or coating paper, wherein paper is treated with an aqueous size or coating liquid that contains the modified starch defined in claim 35.

37. Use of the modified starch defined in claim 35 for surface-  
20 sizing and/or coating paper.

38. Paper obtainable by the process defined in claim 36.

39. Use of maltogenic amylase variant defined in claim 1 for  
25 preparing a modified starch.

40. A food product comprising an emulsion containing the modified starch defined in claim 35.

30 41. A food product according to claim 40, wherein the food product is a beverage.

42. Use of the modified starch as defined in claim 35 for the preparation of a food product.

35

43. A beverage flavour concentrate comprising an emulsion containing the modified starch defined in claim 35.

44. A beverage flavour concentrate according to claim 43, further comprising flavouring oil(s), sweetener(s) and water.

45. Use of the modified starch defined in claim 35 for the  
5 preparation of a beverage flavour concentrate.

46. A flavouring agent comprising an emulsion containing the modified starch defined in claim 35.

10 47. Use of the modified starch as defined in claim 35 for the preparation of a beverage flavour agent.

1/2

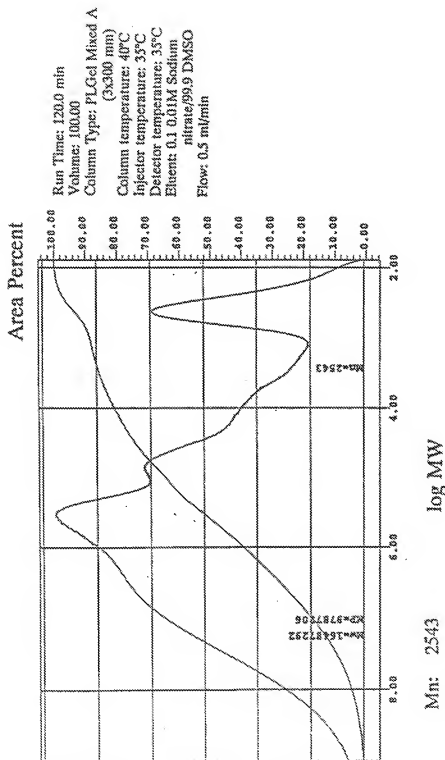


Fig. 1

2/2

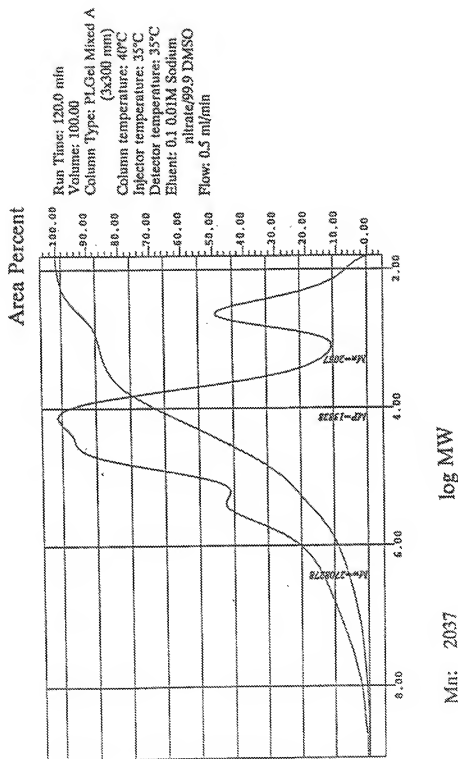


Fig. 2

Mn: 2037  
MP: 13828  
Mw: 2708278

## SEQUENCE LISTING

&lt;110&gt; Novo Nordisk A/S

&lt;120&gt; Method for production of maltose and/or enzymatically modified starch

&lt;130&gt; 5039.204

&lt;140&gt;

&lt;141&gt;

&lt;160&gt; 14

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 2160

&lt;212&gt; DNA

&lt;213&gt; Bacillus sp.

&lt;220&gt;

&lt;221&gt; mat\_peptide

&lt;222&gt; (100)..(2157)

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(2160)

&lt;400&gt; 1

atg	aaa	aag	aaa	acg	ctt	tct	tta	ttt	gtg	gga	ctg	atg	ctc	ctc	atc	48
Met	Lys	Lys	Lys	Thr	Leu	Ser	Leu	Phe	Val	Gly	Leu	Met	Leu	Leu	Ile	
				-30				-25							-20	

ggc	ctt	ctg	ttc	agc	ggc	tct	ctt	cgc	tac	aat	cca	aac	gcc	gct	gaa	96
Gly	Leu	Leu	Phe	Ser	Gly	Ser	Leu	Pro	Tyr	Asn	Pro	Asn	Ala	Ala	Glu	
			-15				-10					-5				

gcc	agc	agt	tcc	gca	agc	gtc	aaa	ggg	gac	gtg	att	tac	cag	att	atc	144
Ala	Ser	Ser	Ser	Ala	Ser	Val	Lys	Gly	Asp	Val	Ile	Tyr	Gln	Ile	Ile	
	-1	1			5				10					15		

att	gac	cgg	ttt	tac	gat	ggg	gac	acg	acg	aac	aac	aat	cct	gcc	aaa	192
Ile	Asp	Arg	Phe	Tyr	Asp	Gly	Asp	Thr	Thr	Asn	Asn	Asn	Pro	Ala	Lys	
				20				25					30			

agt	tat	gga	ctt	tac	gat	cgc	acc	aaa	tcg	aag	tgg	aaa	atg	tat	tgg	240
Ser	Tyr	Gly	Leu	Tyr	Asp	Pro	Thr	Lys	Ser	Lys	Trp	Lys	Met	Tyr	Trp	

35	40	45	
ggc ggg gat ctg gag ggg gtt cgt caa aaa ctt cct tat ctt aaa cag	288		
Gly Gly Asp Leu Glu Gly Val Arg Gln Lys Leu Pro Tyr Leu Lys Gln			
50	55	60	
ctg ggc gta acg aca atc tgg ttg tcc ccg gtt ttg gac aat ctg gat	336		
Leu Gly Val Thr Thr Ile Trp Leu Ser Pro Val Leu Asp Asn Leu Asp			
65	70	75	
aca ctg gcg ggc acc gat aac acg ggc tat cac gga tac tgg acg cgc	384		
Thr Leu Ala Gly Thr Asp Asn Thr Gly Tyr His Gly Tyr Trp Thr Arg			
80	85	90	95
gat ttt aaa cag att gag gaa cat ttc ggg aat tgg acc aca ttt gac	432		
Asp Phe Lys Gln Ile Glu Glu His Phe Gly Asn Trp Thr Thr Phe Asp			
100	105	110	
acg ttg gtc aat gat gct cac caa aac gga atc aag gcg att gtc gac	480		
Thr Leu Val Asn Asp Ala His Gln Asn Gly Ile Lys Val Ile Val Asp			
115	120	125	
ttt gtg ccc aat cat tcg act cct ttt aag gca aac gat tcc acc ttt	528		
Phe Val Pro Asn His Ser Thr Pro Phe Lys Ala Asn Asp Ser Thr Phe			
130	135	140	
ggc gaa ggc ggc gcc ctc tac aac aat gga acc tat atg ggc aat tat	576		
Ala Glu Gly Gly Ala Leu Tyr Asn Asn Gly Thr Tyr Met Gly Asn Tyr			
145	150	155	
ttt gat gac gca aca aaa ggg tac ttc cac cat aat ggg gac atc agc	624		
Phe Asp Asp Ala Thr Lys Gly Tyr Phe His His Asn Gly Asp Ile Ser			
160	165	170	175
aac tgg gac gac cgg tac gag gcg caa tgg aaa aac ttc acg gat cca	672		
Asn Trp Asp Asp Arg Tyr Glu Ala Gln Trp Lys Asn Phe Thr Asp Pro			
180	185	190	
gcc ggt ttc tcg ctt gcc gat ttg tcg cag gaa aat ggc acg att gct	720		
Ala Gly Phe Ser Leu Ala Asp Leu Ser Gln Glu Asn Gly Thr Ile Ala			
195	200	205	
caa tac ctg acc gat gcg gcg gtt caa ttg gta gca car gga gcg gat	768		
Gln Tyr Leu Thr Asp Ala Ala Val Gln Leu Val Ala His Gly Ala Asp			
210	215	220	
ggc ttg cgg att gat gcg gtc aag cat ttt aat tcg ggg ttc tcc aaa	816		
Gly Leu Arg Ile Asp Ala Val Lys His Phe Asn Ser Gly Phe Ser Lys			

225	230	235	
tcg ttg gcc gat aaa ctg tac caa aag aaa gac att ttc ctg gtg ggg			864
Ser Leu Ala Asp Lys Leu Tyr Gln Lys Lys Asp Ile Phe Leu Val Gly			
240	245	250	255
gaa tgg tac gga gat gac ccc gga aca gcc aat cat ctg gaa aag gtc			912
Glu Trp Tyr Gly Asp Asp Pro Gly Thr Ala Asn His Leu Glu Lys Val			
260	265	270	
cgg tac gcc aac aac agc ggt grc aat gtg ctg gat ttt gat ctc aac			960
Arg Tyr Ala Asn Asn Ser Gly Val Asn Val Leu Asp Phe Asp Leu Asn			
275	280	285	
acg gtg att cga aat gtg ttc ggc aca ttt acg caa acg atg tac gat			1008
Thr Val Ile Arg Asn Val Phe Gly Thr Phe Thr Gln Thr Met Tyr Asp			
290	295	300	
ctt aac aat atg gtg aac caa acg ggg aac gag tac aza tac aaa gaa			1056
Leu Asn Asn Met Val Asn Gln Thr Gly Asn Glu Tyr Lys Tyr Lys Glu			
305	310	315	
aat cta atc aca ttt atc gat aac cat gat atg tca aga ttt ctt tcg			1104
Asn Leu Ile Thr Phe Ile Asp Asn His Asp Met Ser Arg Phe Leu Ser			
320	325	330	335
gta aat tcg aac aag gcg aat ttg cac cag gcg ctt gct ttc att ctc			1152
Val Asn Ser Asn Lys Ala Asn Leu His Gln Ala Leu Ala Phe Ile Leu			
340	345	350	
act tcg cgg ggt acg ccc tcc atc tat tat gga acc gaa caa tac atg			1200
Thr Ser Arg Gly Thr Pro Ser Ile Tyr Tyr Gly Thr Glu Gln Tyr Met			
355	360	365	
gca ggc ggc aat gac cgg tac aac cgg ggg atg atg ccg gcg ttt gat			1248
Ala Gly Gly Asn Asp Pro Tyr Asn Arg Gly Met Met Pro Ala Phe Asp			
370	375	380	
acg aca acc acc gcc ttt aaa gag gtg tca act ctg gcg ggg ttg cgc			1296
Thr Thr Thr Thr Ala Phe Lys Glu Val Ser Thr Leu Ala Gly Leu Arg			
385	390	395	
agg aac aat gcg gcg atc cag tac ggc acc acc acc cag cgt tgg atc			1344
Arg Asn Asn Ala Ala Ile Gln Tyr Gly Thr Thr Thr Gln Arg Trp Ile			
400	405	410	415
aac aat gat gtt tac att tat gaa cgg aaa ttt ttc aac gat gtc gtg			1392
Asn Asn Asp Val Tyr Ile Tyr Glu Arg Lys Phe Phe Asn Asp Val Val			

420	425	430	
ttg gtg gcc atc aat cga aac acg caa tcc tcc tat tgg att tcc ggt			1440
Leu Val Ala Ile Asn Arg Asn Thr Gln Ser Ser Tyr Ser Ile Ser Gly			
435	440	445	
ttg cag acg gcc ttg cca aat ggc agc tat gcg gat tat ctg tca ggg			1488
Leu Gln Thr Ala Leu Pro Asn Gly Ser Tyr Ala Asp Tyr Leu Ser Gly			
450	455	460	
ctg ttg ggg ggg aac ggg att tcc gtt tcc aat gga agt gtc gct tgg			1536
Leu Leu Gly Gly Asn Gly Ile Ser Val Ser Asn Gly Ser Val Ala Ser			
465	470	475	
tcc acg ctt gcg cct gga gcc gtg tct gtt tgg cag tac agc aca tcc			1584
Phe Thr Leu Ala Pro Gly Ala Val Ser Val Trp Gln Tyr Ser Thr Ser			
480	485	490	495
gct tca gcg ccg caa atc gga tgg gtt gct cca aat atg ggg att ccg			1632
Ala Ser Ala Pro Gln Ile Gly Ser Val Ala Pro Asn Met Gly Ile Pro			
500	505	510	
ggt aat gtg gtc acg atc gac ggg aaa ggt ttt ggg acg acg cag gga			1680
Gly Asn Val Val Thr Ile Asp Gly Lys Gly Phe Gly Thr Thr Gln Gly			
515	520	525	
acc gtg aca ttt ggc gga gtg aca gcg act gtg aaa tcc tgg aca tcc			1728
Thr Val Thr Phe Gly Gly Val Thr Ala Thr Val Lys Ser Trp Thr Ser			
530	535	540	
aat cgg att gaa gtg tac gtt ccc aac atg gcc gcc ggg ctg acc gat			1776
Asn Arg Ile Glu Val Tyr Val Pro Asn Met Ala Ala Gly Leu Thr Asp			
545	550	555	
gtg aaa gtc acc gcg ggt gga gtt tcc agc aat ctg tat tct tac aat			1824
Val Lys Val Thr Ala Gly Gly Val Ser Ser Asn Leu Tyr Ser Tyr Asn			
560	565	570	575
att ttg agt gga acg cag aca tgg gtt gtg ttt act gtg aaa agt gcg			1872
Ile Leu Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala			
580	585	590	
cct ccg acc aac ctg ggg gat aag att tac ctg acg ggc aac ata ccg			1920
Pro Pro Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro			
595	600	605	
gaa ttg ggg aat tgg agc acg gat acg agc gga gcc gtt aac aat gcg			1968
Glu Leu Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala			

610	615	620	
caa ggg ccc ctg ctc gcg ccc aat tat ccg gat tgg ttt tat gta ttc			2015
Gln Gly Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe			
625	630	635	
agc gtt cca gca gga aag acg att caa ttc aag ttc ttc stc aag cgt			2064
Ser Val Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg			
640	645	650	655
gcg gat gga acg att caa tgg gag aat ggt tcy aac cac gtg gcc aca			2112
Ala Asp Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr			
660	665	670	
act ccc acg ggt gca acc ggt aac att act gtt acg tgg caa aac tag			2160
Thr Pro Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn			
675	680	685	
 <210> 2			
<211> 719			
<212> PRT			
<213> Bacillus sp.			
 <400> 2			
Met Lys Lys Lys Thr Leu Ser Leu Phe Val Gly Leu Met Leu Leu Ile			
1	5	10	15
Gly Leu Leu Phe Ser Gly Ser Leu Pro Tyr Asn Pro Asn Ala Ala Glu			
20	25	30	
Ala Ser Ser Ser Ala Ser Val Lys Gly Asp Val Ile Tyr Gln Ile Ile			
35	40	45	
Ile Asp Arg Phe Tyr Asp Gly Asp Thr Thr Asn Asn Asn Pro Ala Lys			
50	55	60	
Ser Tyr Gly Leu Tyr Asp Pro Thr Lys Ser Lys Trp Lys Met Tyr Trp			
65	70	75	80
Gly Gly Asp Leu Glu Gly Val Arg Gln Lys Leu Pro Tyr Leu Lys Gln			
85	90	95	
Leu Gly Val Thr Thr Ile Trp Leu Ser Pro Val Leu Asp Asn Leu Asp			
100	105	110	
Thr Leu Ala Gly Thr Asp Asn Thr Gly Tyr His Gly Tyr Trp Thr Arg			
115	120	125	

Asp Phe Lys Gln Ile Glu Glu His Phe Gly Asn Trp Thr Thr Phe Asp  
 130 135 140  
 Thr Leu Val Asn Asp Ala His Gln Asn Gly Ile Lys Val Ile Val Asp  
 145 150 155 160  
 Phe Val Pro Asn His Ser Thr Pro Phe Lys Ala Asn Asp Ser Thr Phe  
 165 170 175  
 Ala Glu Gly Gly Ala Leu Tyr Asn Asn Gly Thr Tyr Met Gly Asn Tyr  
 180 185 190  
 Phe Asp Asp Ala Thr Lys Gly Tyr Phe His His Asn Gly Asp Ile Ser  
 195 200 205  
 Asn Trp Asp Asp Arg Tyr Glu Ala Gln Trp Lys Asn Phe Thr Asp Pro  
 210 215 220  
 Ala Gly Phe Ser Leu Ala Asp Leu Ser Gln Glu Asn Gly Thr Ile Ala  
 225 230 235 240  
 Gln Tyr Leu Thr Asp Ala Ala Val Gln Leu Val Ala His Gly Ala Asp  
 245 250 255  
 Gly Leu Arg Ile Asp Ala Val Lys His Phe Asn Ser Gly Phe Ser Lys  
 260 265 270  
 Ser Leu Ala Asp Lys Leu Tyr Gln Lys Lys Asp Ile Phe Leu Val Gly  
 275 280 285  
 Glu Trp Tyr Gly Asp Asp Pro Gly Thr Ala Asn His Leu Glu Lys Val  
 290 295 300  
 Arg Tyr Ala Asn Asn Ser Gly Val Asn Val Leu Asp Phe Asp Leu Asn  
 305 310 315 320  
 Thr Val Ile Arg Asn Val Phe Gly Thr Phe Thr Gln Thr Met Tyr Asp  
 325 330 335  
 Leu Asn Asn Met Val Asn Gln Thr Gly Asn Glu Tyr Lys Tyr Lys Glu  
 340 345 350  
 Asn Leu Ile Thr Phe Ile Asp Asn His Asp Met Ser Arg Phe Leu Ser  
 355 360 365  
 Val Asn Ser Asn Lys Ala Asn Leu His Gln Ala Leu Ala Phe Ile Leu  
 370 375 380

Thr Ser Arg Gly Thr Pro Ser Ile Tyr Tyr Gly Thr Glu Gln Tyr Met  
 365 390 395 400  
 Ala Gly Gly Asn Asp Pro Tyr Asn Arg Gly Met Met Pro Ala Phe Asp  
 405 410 415  
 Thr Thr Thr Thr Ala Phe Lys Glu Val Ser Thr Leu Ala Gly Leu Arg  
 420 425 430  
 Arg Asn Asn Ala Ala Ile Gln Tyr Gly Thr Thr Thr Gln Arg Trp Ile  
 435 440 445  
 Asn Asn Asp Val Tyr Ile Tyr Glu Arg Lys Phe Phe Asn Asp Val Val  
 450 455 460  
 Leu Val Ala Ile Asn Arg Asn Thr Gln Ser Ser Tyr Ser Ile Ser Gly  
 465 470 475 480  
 Leu Gln Thr Ala Leu Pro Asn Gly Ser Tyr Ala Asp Tyr Leu Ser Gly  
 485 490 495  
 Leu Leu Gly Gly Asn Gly Ile Ser Val Ser Asn Gly Ser Val Ala Ser  
 500 505 510  
 Phe Thr Leu Ala Pro Gly Ala Val Ser Val Trp Gln Tyr Ser Thr Ser  
 515 520 525  
 Ala Ser Ala Pro Gln Ile Gly Ser Val Ala Pro Asn Met Gly Ile Pro  
 530 535 540  
 Gly Asn Val Val Thr Ile Asp Gly Lys Gly Phe Gly Thr Thr Gln Gly  
 545 550 555 560  
 Thr Val Thr Phe Gly Gly Val Thr Ala Thr Val Lys Ser Trp Thr Ser  
 565 570 575  
 Asn Arg Ile Glu Val Tyr Val Pro Asn Met Ala Ala Gly Leu Thr Asp  
 580 585 590  
 Val Lys Val Thr Ala Gly Gly Val Ser Ser Asn Leu Tyr Ser Tyr Asn  
 595 600 605  
 Ile Leu Ser Gly Thr Gln Thr Ser Val Val Phe Thr Val Lys Ser Ala  
 610 615 620  
 Pro Pro Thr Asn Leu Gly Asp Lys Ile Tyr Leu Thr Gly Asn Ile Pro  
 625 630 635 640

Glu Leu Gly Asn Trp Ser Thr Asp Thr Ser Gly Ala Val Asn Asn Ala  
                   645                                  650                                  655  
 Gln Gly Pro Leu Leu Ala Pro Asn Tyr Pro Asp Trp Phe Tyr Val Phe  
                   660                                  665                                  670  
 Ser Val Pro Ala Gly Lys Thr Ile Gln Phe Lys Phe Phe Ile Lys Arg  
                   675                                  680                                  685  
 Ala Asp Gly Thr Ile Gln Trp Glu Asn Gly Ser Asn His Val Ala Thr  
                   690                                  695                                  700  
 Thr Pro Thr Gly Ala Thr Gly Asn Ile Thr Val Thr Trp Gln Asn  
                   705                                  710                                  715

&lt;210&gt; 3

&lt;211&gt; 38

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: F 188H Primer

&lt;400&gt; 3

gcaatggaaa aaccacacgg atccagccgg cttctcgc 38

&lt;210&gt; 4

&lt;211&gt; 38

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: F 188 E Primer

&lt;400&gt; 4

gcaatggaaa aacgagacgg atccagccgg cttctcgc 38

&lt;210&gt; 5

&lt;211&gt; 36

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: F 284E Primer

<400> 5

gggtgtcaatg tgcgggatga agatctcaac acgggtg

36

<210> 6

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: F 284D Primer

<400> 6

ggtgtcaatg ttctagatga tgatctcaac acgggtg

36

<210> 7

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: F 284K Primer

<400> 7

ggtgtcaatg tgcgggataa agatctcaac acgggtg

36

<210> 8

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: N 327D Primer

<400> 8

cacatttacc gatgatcatg atgtgtcaag atttc

35

<210> 9

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: T 288K Primer

<400> 9

cctaaaaacta gagttgttcc actaggcctt acac

34

<210> 10

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: T 288R Primer

<400> 10

cctaaaaacta gagttgtccc actaggcctt acac

34

<210> 11

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: A 189 Primer

<400> 11

tgggcaatta ttttgatgac gc

22

<210> 12

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: B 649 Primer

<400> 12

tccgctcgta tccgtgctcc

20

<210> 13

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: A62 Primer

<400> 13

ggggatcttg agggggttcg

20

<210> 14

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: B 346 Primer

<400> 14

tttgtaactcg ttcccggtt gg

22

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 00/00461

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12P 19/18, C12N 9/26 // A23L 1/09, D21H 19/54

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12P, C08B, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9943794 A1 (NOVO NORDISK A/S), 2 Sept 1999 (02.09.99)  ---	1-47
P,X	WO 9943793 A1 (NOVO NORDISK A/S), 2 Sept 1999 (02.09.99), & Geneseq database accession no. Y31731 100% identity in 719 aa overlap  ---	1-47
X	Database Swissprot, Accession no. P19531, 1991-02-01, 97.9% identity in 721 aa overlap & FEHS Microbiology Letters 56, 53-60 (1988), Diderichsen B et al: "Cloning of a maltogenic alpha-amylase from Bacillus"  ---	1-17

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier application or patent published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is considered with one or more other such documents, such combination being obvious to a person skilled in the art

\*Z\* document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

8 January 2001

11-01-2001

Name and mailing address of the ISA/

Authorized officer

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Yvonne Stösteen/EO

Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 00/00461

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0120693 A1 (NOVO INDUSTRI A/S), 3 October 1984 (03.10.84) ---	1-47
X	WO 9510627 A1 (NOVO NORDISK A/S), 20 April 1995 (20.04.95) ---	1-47
Y	WO 9743424 A1 (GENENCOR INTERNATIONAL, INC.), 20 November 1997 (20.11.97) ---	18-47
Y	FEBS Letters, Volume 456, 1999, Stefan Janecek et al, "The evolution of starch-binding domain" page 119 - page 125 ---	18-47
A	Starch/Stärke, Volume 50, No 1, 1998, Claus Christophersen et al, "Enzymatic Characterisation of Novamyl, a Thermostable alpha-Amylase" page 39 - page 45 ---	1-47
A	WO 9633267 A1 (NOVO NORDISK A/S), 24 October 1996 (24.10.96) ---	18-47

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK00/00461**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: **1 partially**  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
**see next sheet**
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

**see next sheet**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## Box I.2

Present claim 1 is constructed in such a complex way and being so broad and speculative that it is not possible to carry out a meaningful complete search. See Art 17(2)(a).

Claim 1 fails to characterize the used variants in a way which is clear and concise. The expression "has at least 70% identity to position 1-686 of SEQ ID NO:1" combined with pH optimum of 3.5-7 do not define the desired characteristics and properties of the enzyme in a definite way. The claim so lacks support within the meaning of Article 6 PCT.

The search has mainly been carried out for a method using thermostable mutated variants of maltogenic amylase, where the parent enzyme has the amino acid sequence shown in position 1-686 of SEQ ID NO:1.

Box II

Lack of unity of invention:

The International Searching Authority is of the opinion that the present application lacks unity (rule 13.1 PCT). It is considered that at least 8 different inventions are claimed for in this application. Each invention relates to methods using variants of the maltogenic amylase having the amino acid sequence SEQ ID NO:1. Since each mutation also can be seen as a separate invention there would be a huge number of inventions.

The observation is based on the following reasons:

The inventions:

The inventions relate to methods for preparing maltose and /or modified starch by treating starch with variants of a maltogenic amylase. The description indicates that the variants should be characterized by having improved stability especially by having improved thermostability.

The Prior art:

The thermostable maltogenic amylase from *Bacillus* of the present application is disclosed in EP 0120693. It is most active at 60-70°C and is frequently used in the starch industry.

The sequence of the similar maltogenic amylase is known from the Swissprot database, accession no P19531, 01 feb 1991, Diderichsen et al. The amino acid sequence has 97.9% identity with the amino acid sequence of the enzyme (SEQ ID NO:1) of the present claims.

The preparation of thermostable maltogenic amylase variants is made by well-known random mutagenesis methods.

Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"-i.e. features that define a contribution which each of the inventions makes over the prior art. (See Rule 13.2 PCT)

It is not considered that the preparation and use of a variant, with improved stability, of the known maltogenic

.../...

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK00/00461

amylase when preparing maltose and/or modified starch is considered to make a contribution over prior art and is therefore not considered to constitute such "special technical feature".

Consequently there is no single inventive concept underlying the different claimed inventions and thus lack of unity within the sense of Art 17(3) (a) ECT.

In the light of the prior art the present application presents at least the following inventions:

- 1.A method for preparing maltose and/or modified starch by treating starch with thermostable variants of the maltogenic amylase (SEQ ID NO:1) according to claims 18 and 28. These variants are prepared by filling or reducing the size of internal cavities and crevices, e.g. by introducing one or more hydrophobic contacts, preferably by introducing amino acids with bulkier side groups in the vicinity or surroundings of the cavity.
- 2.A method by treating starch with variants having an altered stability due to an altered stabilization of calcium ( $Ca^{2+}$ ) binding according to claim 24.
- 3.A method by treating starch with variants having improved stability by the introduction of one or more interdomain disulfide bonds according to claim 27.
- 4.A method by treating starch with variants having improved stability and on or more salt bridges as compared to said parent enzyme. Mutations at positions N106, N320 and Q624 according to claim 29.
- 5.A method where the variants used lack a deamination site according to claim 25.
6. A method where the variants used have an altered charge distribution according to claim 30.
7. A method where the variants used have an altered domain interaction according to claim 32.

Other methods using other variants mentioned in e.g. claims 18, 19, 21, 22, 23, 33, 34 etc can also be considered as separate inventions.

As all claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fee.

INTERNATIONAL SEARCH REPORT  
Information on patent family members

04/12/00

International application No.

PCT/DK 00/00461

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9943794	A1	02/09/99	AU	2512899 A	15/09/99
				AU	2512999 A	15/09/99
				BR	9908281 A	31/10/00
				WO	9943793 A	02/09/99
WO	9943793	A1	02/09/99	AU	2512899 A	15/09/99
				AU	2512999 A	15/09/99
				BR	9908281 A	31/10/00
				WO	9943794 A	02/09/99
EP	0120693	A1	03/10/84	SE	0120693 T3	
				AT	43634 T	15/06/89
				CA	1214407 A	25/11/86
				DE	3478469 D	00/00/00
				DK	135983 D	00/00/00
				DK	153799 B,C	05/09/88
				DK	161084 A	26/09/84
				JP	1786423 C	10/09/93
				JP	4072505 B	18/11/92
				JP	60002185 A	08/01/85
				US	4598048 A	01/07/86
				US	4604355 A	05/08/86
WO	9510627	A1	20/04/95	AU	7853294 A	04/05/95
				DK	114893 D	00/00/00
WO	9743424	A1	20/11/97	AU	2996997 A	05/12/97
				BR	9709088 A	03/08/99
				EP	0927259 A	07/07/99
				JP	2000509995 T	08/08/00
				US	5763385 A	09/06/98
WO	9633267	A1	24/10/96	AU	5396896 A	07/11/96
				CA	2217876 A	24/10/96
				EP	0822982 A	11/02/98
				JP	11503906 T	06/04/99
				US	6004790 A	21/12/99